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THE UNIVERSITY OF ALBERTA

Effect of Endurance Type Exercise (ETE) Upon Selected Ca^{2+} Transport Parameters From Rat Sarcoplasmic Reticulum

by

James S. C. Gilchrist

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

Department of Physical Education and Sport Studies

Edmonton, Alberta

SPRING, 1986

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Effect of Enduranee Type Exercise (ETE) Upon Selected Ca^{2+} Transport Parameters From Rat Sarcoplasmic Reticulum submitted by James S. C. Gilchrist in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE.

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Date 22nd April 1986.

Abstract

The present study examined the response of several Ca^{2+} transport parameters to exhaustive treadmill running in purified native sarcoplasmic reticulum (SR) vesicles isolated from rat gastrocnemius muscle. Ca^{2+}-loading and anion-induced Ca^{2+}-release was unaltered ($p>0.05$) when the exhausted (E) condition was compared to controls (C). The consistent release of Ca^{2+} in sucrose incubated vesicles suggests that this mechanism of Ca^{2+} release is not entirely osmotic in nature. Initial rate and capacity for oxalate free Ca^{2+}-uptake were elevated ($p<0.05$) in E when compared to C. Peak uptake values were 52.32 ± 5.8 and 38.56 ± 1.2 nmol Ca^{2+}.mg SR^{-1} (mean $\pm$ sem) for E and C respectively. On the other hand the Km and Vmax of ATP hydrolysis were depressed by approximately 18% in E when the data was expressed as a linear Hoftsee plot. The changes in kinetic parameters could not be attributed to an altered SR protein profile since SDS-PAGE showed no differences between C and E. These data suggest that Ca^{2+}-ATPase function, in vitro, may be potentiated rather than depressed in view of the apparent reduction in energy cost of Ca^{2+} transport. This may reflect an in vivo accomodation by the Ca^{2+}-ATPase to an exercise induced perturbation of the intracellular ionic and energy states.

Acknowledgements

When I began this work I had hoped that by the time I reached this point I would not feel haunted by hindsight. Given several limitations (particularly my own) I feel that this work, while hardly a citation classic, represents a significant personal milestone in terms of both knowledge and maturity. It is a milestone I once dared not believe I could achieve and for that I owe immeasurably to several key individuals.

The final production of this work is, in itself, no simple task. For her devotion, industry, and fathomless capacity to endure my tyrannical outbursts I would like to thank my fiancee Jo-Anne Flory. I love her very much and could not have arrived at this point without her.

I would like to thank also those many individuals, particularly my friends and colleagues in the lab, whose incidental efforts and thoughts have been invaluable.

Finally, but most importantly, I would like to thank my advisor Dr. Angelo Belcastro. His seemingly boundless perception, inspiration, and direction have meant just about everything as far as this work and my professional experience in Edmonton are concerned. I cannot say enough for the support and generosity he has shown me "both on and off the field". He has been both mentor and contemporary; leader and very dear friend. Thankyou for not allowing me to feel haunted by hindsight.

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CHAPTER 1

Introduction

Muscle fatigue has been defined as "the inability of a physiological process to continue functioning at a particular level and/or the inability of the total organism to maintain a pre-determined work intensity" (Edwards, 1983). Mechanically, muscle fatigue and/or exhaustion is usually characterized by decreases in peak tetanic tension, the rates of tension development and relaxation, and increases in twitch contraction time and half relaxation time (Dawson et al., 1980). These observations are reported for both chronically tetanised isolated muscle preparations (Petrofsky and Fitch, 1980) and in muscles from rats swum to exhaustion (Fitts et al., 1979, 1982). Petrofsky and Fitch (1980) also demonstrated a reduced maximum rate of shortening although this parameter was unaffected (Fitts et al., 1977) where the muscles were excised from trained animals.

Since the earlier investigations of Hill (1928), muscle fatigue has been the focus of considerable research interest. Yet, although numerous biochemical and physiological observations have been linked with the fatigue process, its onset to the point of muscle exhaustion remains to be adequately explained through cause-effect relationships. Earlier reports (Ikai and Steinhaus, 1961; Stevens and Taylor, 1972) proposed that fatigue was associated with aberrant neural and neuromuscular transmission. The validity of these studies was later challenged on the basis of methodological inconsistencies (Fink and Luttgau, 1976). More recently, the determinants of fatigue are suggested to be, primarily, localised intramuscularly since fatigue can be induced in directly stimulated muscles (Edgerton et al., 1980; Petrofsky et al., 1980).

In order to account, intramuscularly, for the mechanical manifestations of fatigue, several key subcellular processes have been offered as explanations. These include, the role of

substrate production/availability, the role of force generation/transmission at the level of the myofibrils, and the role of excitation-contraction (E-C) coupling. With respect to the former possibility, the mitochondria do not appear to be involved directly, since the capacity for oxidative phosphorylation is unaltered in chronically exhausted skeletal and cardiac muscle (Tomanek and Banister, 1972; Terjung et al., 1972). Alternatively, suboptimal oxidative phosphorylation, in vivo, was suggested to occur as a consequence of depleted muscle glycogen stores that often characterize chronically exhausted muscle models (Terjung et al., 1972; Gollnick et al., 1974). To date, however, the role of substrate availability during the onset of exhaustion remains unresolved since its importance may be dependent upon the nature of the exercise protocol and muscle fibre type (Dobson et al., 1985; Parkhouse et al., 1985). In endurance type exercise (ETE) protocols, substrate availability may not, necessarily, be a primary limiting factor since (a) intramuscular ATP content may be unaltered at exhaustion (Dawson et al., 1980) and (b) glycogen depleted muscles can retain their normal contractile characteristics (Fitts et al., 1982).

Recent evidence strongly implicates the myofibrils, directly, in the expression of ETE fatigue (Belcastro et al., 1984, 1985). Although the Ca^{2+} dependency of the myofibrillar ATPase, as assessed from Hill type kinetics, is unaltered at exhaustion (Belcastro et al., 1984; Fitts et al., 1982), electron micrographs display significant disruption of the myofilament ultrastructure and Z-line integrity (Belcastro et al., 1985). The effects observed for ETE fatigue are very similar to the effect of raised intracellular Ca^{2+} upon myofilament ultrastructure (Duncan et al., 1980). In this regard, cell calcium imbalance after chronic stimulation has been indicated from cryo-ultramicrotomy studies of intracellular elemental Ca^{2+} composition (Gonzalez-Serratos et al., 1978; Sembrowich et al., 1983). Consequently, Belcastro et al.(1985) postulated that depressed force generation of the whole muscle at exhaustion may be a reflection of depressed force transmission between sarcomere units due to myofilament ultrastructural disruption. This course of events, in turn, may be precipitated by a putative ETE induced cell calcium overload. Accordingly the sarcoplasmic reticulum (SR), which

regulates myoplasmic Ca^{2+} concentrations, may be instrumental in this effect.

Earlier studies have implicated the Ca^{2+} stimulated-Mg^{2+} dependent SR ATPase (Ca^{2+}-ATPase), which mediates Ca^{2+} sequestration and hence relaxation, in the expression of muscle fatigue. The observed depression (20%) in oxalate supported Ca^{2+} accumulation, defined as Ca^{2+}-loading, (Sembrowich and Gollnick, 1977; Fitts et al., 1982) and Ca^{2+}-ATPase mediated ATP hydrolysis (Hashimoto et al., 1978; Belcastro et al., 1981) by native SR vesicles from rats chronically exercised to exhaustion was suggested to account for the depressed relaxation rates. However, interpretation of these data is limited since the observed depression in Ca^{2+}-loading might be a consequence of a ETE induced increase in membrane leakiness to Ca^{2+}. Feher and Briggs (1980) emphasised that the diagnostic value of Ca^{2+}-loading as a measure of unidirectional Ca^{2+} influx is questionable since they showed that the rate and extent of calcium oxalate seeding and precipation, respectively, are dependent upon vesicle permeability to Ca^{2+}. Consequently, the depressed Ca^{2+}-loading observed by Fitts et al. (1982) might not reflect a dysfunctional Ca^{2+}-ATPase but, rather, increased membrane leakiness to Ca^{2+}, particularily as they found concomittant ATP hydrolysis to be unaltered at exhaustion. Furthermore, these fatigue related studies of Ca^{2+} transport were performed upon crude homogenates with no account taken of the microsomal protein profile. Crude SR preparations are subject to contamination from exogenous proteins, in particular phosphorylase b (MacLennan, 1970). This contamination will compound the variability of the Ca^{2+}-ATPase and, possibly, bias the Ca^{2+}-loading and ATPase data. Little confidence, therefore, can be ascribed to prior interpretations of alterations in Ca^{2+}-loading if the population of the Ca^{2+}-ATPase, in relation to the total SR protein yield, differs between control and experimental conditions. In addition, an endpoint determination of inorganic phosphate (Pi) at a single and relatively high concentration of Mg·ATP (5mM) does not, as an assay of Ca^{2+}-ATPase activity, adequately reflect the catalytic potential of the SR Ca^{2+} pump in a microsomal preparation. Unlike the detergent solubilised preparation, the vesicular form of the Ca^{2+}-ATPase is regulated by osmotic and Ca^{2+} electrochemical gradients, variations in which will alter net Pi production

(Hasselbach 1979; Hasselbach et al., 1981; Hasselbach and Migala, 1985). Such colorimetric determinations of Pi have formed the basis of all previous ETE related studies upon Ca^{2+}-ATPase activity. The generalisability of the subsequent results is limited to a description of ATP regulation at the Ca^{2+}-ATPase low affinity substrate binding site. The catalytic potential of the Ca^{2+}-ATPase is more appropriately studied in terms of the substrate dependency at lower concentrations of Mg·ATP (Moller et al., 1980).

A more comprehensive physiological role of SR Ca^{2+} transport during ETE may be appreciated when account is taken of both Ca^{2+} influx and efflux and considered in relation to ATPase activity. Although Ca^{2+}-loading may be a useful parameter, some assesment of potential ETE effects upon the efflux component of net Ca^{2+} transport is necessary. This may be achieved through the accumulation of Ca^{2+} in the absence of precipitating anions (Ca^{2+}-uptake) since at steady state Ca^{2+} uptake the rate of Ca^{2+} influx equals the rate of Ca^{2+} efflux (Feher and Briggs, 1983).

The purpose of the present study is, therefore, to re-evaluate the role of the SR during ETE fatigue through determination of 1) Ca^{2+}-loading, 2) Ca^{2+}-uptake and 3) substrate dependent ATPase kinetic profiles using purified native SR vesicles. An ion substitution induced-Ca^{2+} release procedure (Koshita et al., 1982) will also be employed in order to assess the potential effects of ETE fatigue upon a hypothetical Ca^{2+} release mechanism.

CHAPTER 2

Methodology

Animal Care and Exercise Protocol

Ten female Sprague=Dawley rats (210-265g) were randomly assigned to either a non-exercising control (C) group (N=5) or exercise (E) group (N=5). The animals were fed a diet of Purina rat chow and water, ad libitum, and were maintained on a 12 hour reverse day/night cycle at constant temperature. All animals were familiarized with treadmill running 2 days prior to tissue sampling. E animals were run to exhaustion on a motor driven treadmill at an inclination of 10%. The criterion for exhaustion was determined as the inability of animals to avoid the shock grid at the rear of the treadmill and/or to right themselves following exercise.

Tissue Sampling

All animals were sacrificed by initial stunning and subsequent decapitation. Gastrocnemius muscles from both hind limbs were excised and quick frozen in isopentane pre-cooled with liquid nitrogen. The muscles were stored (-70°C) until subsequent biochemical analysis. Both muscles from each animal were removed within 3 minutes after the point of exhaustion. Paired muscles from each animal were pooled to represent a single sample.

Biochemical Analysis

Vesicle Preparation

SR vesicles were isolated according to the procedure Harigaya and Schwartz (1969) as modified by Belcastro et al. (1980). Gastrocnemius muscles were homogenised in a buffer containing (in mM): 100 KCl, 20 Tris-maleate, 1 phenylmethyl sulphonylfluoride (PMSF) (pH 6.8 at 4°C). The homogenate was centrifuged at 1,000xg for 20 minutes to precipitate myofibrils. Subsequent differential centrifugation of aspirated supernatants removed heavy mitochondria at 9,000xg (15 minutes) and light mitochrondria at 15,000xg (20 mins). The final SR containing pellet derived from centrifugation at 37,000xg was washed 3 times in a buffer containing (in mM): 600 KCl, 20 Tris-maleate, 10 NaN_3, 1 PMSF (pH 6.8 at 4°C). Washing was performed with the use of a teflon pestle and glass homogenising tube. The resultant pellet was resuspended finally in a buffer containing (in mM): 100 KCl, 20 tris-maleate (pH 7.0 at 30°C).

The protein content of the final SR suspension was determined by the method of Lowry et al. (1951). Sodium dodecyl sulphate (SDS) was added during the incubation in order to fully solubilise the complex intrinsic membrane Ca^{2+}-ATPase protein.

ATPase Activity

$Ca^{2+} + Mg^{2+}$ ATPase activity was determined as follows. SR protein (final concentration = 50μg.ml^{-1}) was preincubated for 3 minutes in a reaction medium containing (in mM): 100 KCl, 40 Tris-maleate, 5 $MgCl_2$, 0.04 $CaCl_2$ (pH 7.0 at 30°C). The addition of varying concentrations of $Mg{\cdot}ATP^{2-}$ initiated the ATPase reaction which was quenched after 10 minutes upon addition of an equal volume of ice cold 12% trichloroacetic acid (TCA). The precipitated protein was sedimented by centrifugation (1,000xg for 10 mins) and the phosphate content determined spectrophotometrically (700nm) as the the reduction of the phosphomolybdate complex in an ammonium molybdate-ferrous sulphate solution (Taussky and Shorr, 1953). The substrate dependency of the ATPase reaction was assessed through varying the substrate

concentration (0.75 to 5mM $Mg{\cdot}ATP^{2-}$).

Ca^{2+} Transport

Several Ca^{2+} transport parameters were measured using millipore filtration. The description of these parameters is in accord with the terminology employed by Meissner (1975). Ca^{2+} actively accumulated by SR vesicles in the presence of ATP and precipitating anions (oxalate) is defined as Ca^{2+}-loading. Ca^{2+}-accumulated in the presence of ATP but without oxalate is referred to as Ca^{2+}-uptake. Ca^{2+} accumulated by the vesicle in the absence of both ATP and oxalate is defined as Ca^{2+}-binding. For Ca^{2+}-uptake and Ca^{2+}-binding, SR vesicles ($25\mu g{\cdot}ml^{-1}$) were pre-incubated in a reaction medium containing (in mM): 100 KCl, 40 Tris-maleate, 5 $MgCl_2$, 0.050 $^{45}CaCl_2$ (10,000 dpm. $nmole^{-1}$) (pH 7.0 at 30°C). Ca^{2+}-uptake was initiated by the addition of 5mM $Mg{\cdot}ATP^{2-}$. After 1, 2 and 3 minutes 500μl aliquots of the reaction medium were vacuum filtered across millipore filters (HAWP 0.45μ). For studies of Ca^{2+}-loading the foregoing procedure was followed except that 5mM sodium oxalate was included in the reaction medium. The filter trapped vesicles were then washed with 2 volumes of ice-cold distilled-deionised H_2O. Although some researchers (Campbell and Shamoo, 1980) commonly employ higher washing volumes (5-10 vol), pilot work indicated that washes greater than 2 volumes did not decrease, further, the non-specifically bound Ca^{2+}. Besides this, non-specifically bound Ca^{2+} is automatically accounted for in the subtraction of Ca^{2+}-binding from gross Ca^{2+}-loading and Ca^{2+}-uptake values. Ice cold H_2O was used as the rinse medium (instead of incubation buffer) in order to minimize potential ion induced Ca^{2+} release effects. This treatment appears justified since a fraction of the Ca^{2+} accumulated is released upon washing with iso-osmotic incubation buffer. Filters were subsequently dried and solubilised in 10ml Bray's scintillation cocktail.

Ca^{2+} Release

For studies of Ca^{2+}-release the procedure of Koshita et al. (1982) was followed. Vesicles were pre-incubated (45 minutes at 25°C) in a buffer containing (in mM) : 200

potassium gluconate, 10 HEPES, 1 $MgCl_2$, 0.05 $^{45}CaCl_2$ (10,000 dpm. $nmole^{-1}$) with or without 200 sucrose (pH 6.8). Vesicles were pre-incubated at 30°C for 3 mins with the reaction initiated by the addition of 5mM $Mg{\cdot}ATP^{2-}$. After 3 minutes a 0.5ml aliquot was vacuum filtered and the vacuum strength (10 cm Hg) was monitored using a simple mercury manometer The filter was then washed with an equal volume of rinse buffer containing (in mM) : 200 potassium gluconate or 200 KCl, 10 HEPES, 1 $MgCl_2$ (pH 6.8). For sucrose containing reaction media the respective rinse buffer also contained sucrose (200 mM). The filters were then dried and solubilised in 10 ml Bray's scintillation cocktail.

Liquid scintillation counting was performed upon a Beckman LS 7800. The absolute values for Ca^{2+}-uptake, Ca^{2+}-loading and Ca^{2+}-binding (nmoles $Ca^{2+}{\cdot}mg$ SR $protein^{-1}$) were calculated in relation to the known specific activities of the respective reaction media. Net values for the first two parameters were then derived by subtraction of Ca^{2+}-binding. The amount of Ca^{2+} released was determined from the difference between residual Ca^{2+} after the filter trapped vesicles were washed with potassium gluconate and KCl containing rinse buffers.

Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was performed on either 7.5 or 12.5% acrylamide gels by the method of Laemmli (1970). The SR fractions were dissolved in 2 volumes of a sample buffer containing: 0.062M tris-SDS stock, 2.5% SDS, 1% glycerol, 0.05M mercaptoethanol and 0.1ml of 1% bromophenol blue. Aliquots (25μg/well) were subjected to electrophoresis on 7.5% or 12.5% resolving gels and a 3% stacking gel. Molecular weight determinations were accomplished from calibration curves using molecular weight standards (Sigma Chemicals). The gels were scanned at 540nm with a Pye-Unicam 8800 series densitometer.

Histochemistry

Glycogen depletion as a result of exercise was evaluated in the whole gastrocnemius muscle using serial sections for a) periodic acid schiff (PAS) stain (Karlsson et al.,1970) and,

b) Myosin ATPase(pH 10.4) (Guth and Samaha, 1969). Frozen serial sections were cut at -22 °C using a microtome in a cryostat. The sections were mounted on cover glasses and air dried for at least 10 hours.

Data Manipulation and Statistical Analysis

Two-way analyses of variance (ANOVA) were conducted upon the data obtained for Ca^{2+}-uptake and Ca^{2+}-loading. Main effects were incubation time (1,2 or 3 mins) and grouping (control vs exhausted). Two-way ANOVAs were also employed for Ca^{2+}-release but main effects were wash media and grouping. Post-hoc Scheffe multiple comparisons of main effects were performed to test for significance among F values. T-tests were used to test for significant differences between C and E groups with respect to Km and Vmax ATPase parameters. All significant differences were determined at a 95% confidence level ($p<0.05$). Linear transformations of the ATPase data (velocity versus substrate concentration) were performed in order to construct linear regression Hoftsee plots. This involved rearrangement of the classic equation for a straight line; $Y = MX + C$

After rearrangement $Y = M_{\circ} - YC.X^{-1}$

In this case

a. Y = enzyme velocity V (ordinate)
b. X^{-1} = substate concentration^{-1}
c. $Y.X^{-1}$ = abscissa
d. M and C = algebraic constants

CHAPTER 3

Results

The running protocol employed in this study (mean run time = 126.6±6.5 minutes) normally results in an approximate 85% depletion in muscle glycogen content (Belcastro and Sopper, 1984). Such a result is indicative of extensive whole muscle recruitment during the particular exercise (Burke and Edgerton, 1975). In the present study whole gastrocnemius muscle glycogen content was not quantitatively assayed in either control (C) or exhausted (E) animals. It is therefore assumed that this running protocol elicited a comparable level of glycogen depletion as observed by Belcastro and Sopper (1984). Assaying for whole muscle glycogen does not, however, describe the extent of individual fibre type recruitment. Mixed fibre muscles such as the gastrocnemius when subjected, chronically, to slowly repetitive contractions exhibit a well characterised pattern of fibre recruitment. Predominantly slow-oxidative fibres are recruited initially whereas towards the latter stage of the contractile period the progressive recruitment of fast-glycolytic fibres achieves prominence (Gollnick, et al., 1974). In order to assess the degree of total muscle fibre recruitment histochemical staining for glycogen (PAS) and myosin ATPase was performed (Plates 1 and 2). Plate 1 shows a representative PAS stain for the control. Comparison with this is made in Plate 2a which shows the comparable stain for the exhausted muscle. Clearly, the exhausted muscle is extensively depleted across all major fibre types as evidenced by the lack of stain. Furthermore, that this depletion is not fibre specific and that the sectioning is representative of the muscle is demonstrated by comparison of Plates 2a and 2b. Plate 2b which is stained for myosin ATPase represents a serial section of Plate 2a with the vascular bundle at the centre of each plate providing a common landmark. The darkly staining fibres in 2b are fast twitch (oxidative and glycolytic) in character. It can be seen that these fibre types correspond to the few more darkly

Plate 1. Transverse section of C Gastrocnemius stained for glycogen with PAS. Intensity of stain is proportional to glycogen content of individual muscle fibres. Variability of staining is due to mixed fibre type population of Gastrocnemius.

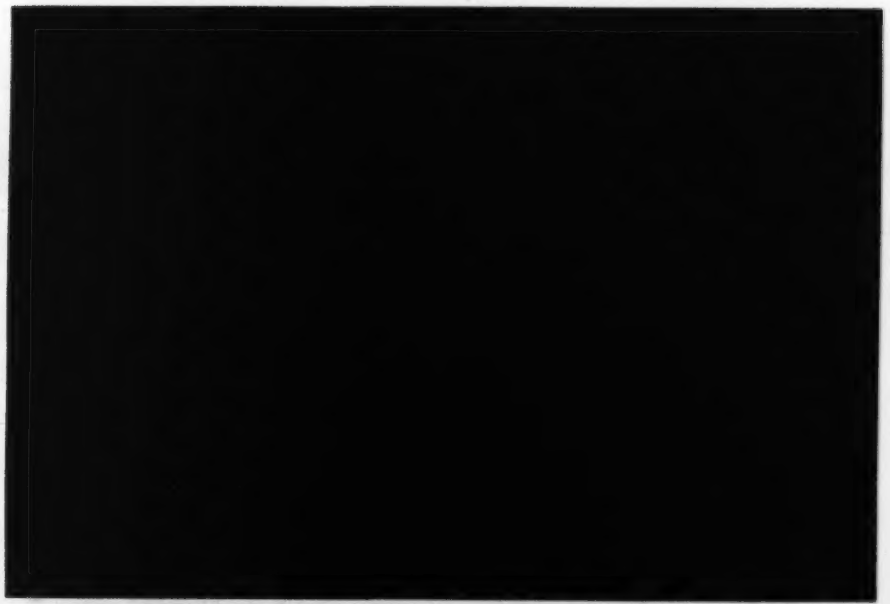

Plate 2a(upper). Transverse section of E Gastrocnemius stained for glycogen with PAS. Uniform lack of stain indicates extensive glycogen depletion across all fibre types.

Plate 2b(lower). Serial section of (a) above stained for myosin ATPase (pH 10.4).Fast twitch fibres (oxidative and glycolytic) stained more darkly than slow twitch fibres.

PAS stained fibres in Plate 2a (to the left of the vascular bundle).

Purified microsomal protein yield (mg SR protein.g muscle^{-1}) did not differ ($p>0.05$) between C and E animals (C=1.44±0.13 ; E=1.42±0.13 (mean±SEM)). Futhermore, the content and relative distribution of the Ca^{2+} transport proteins within the microsomal fraction does not differ between C and E tissue (Figure 3.1). Figure 3.1 is a histogram showing the relative proportions of 4 principle proteins within the microsomal fraction. The major protein, the 100,000 dalton Ca^{2+}-ATPase, is the Ca^{2+} transport pump. The 63,000 dalton protein is Calsequestrin which is involved in Ca^{2+} storage in vivo (Maclennan, 1974). Owing to it's high acidity it migrates anomalously in gel systems at high pH (such as the Laemlli system employed in the present study). Recently, Cozens and Reithmeier (1984) determined that the true molecular weight is approximately 40,000. The 55,000 dalton M_{55} protein is present in the microsomal fraction although it is not considered to be involved in physiological Ca^{2+} transport. The 53,000 dalton band represents a membrane spanning glycoprotein that has specific nucleotide binding characteristics (Campbell and Maclennan, 1983). The histogram was constructed from densiometric scans of SDS gels from each animal microsomal fraction. Plate 3 is an example of such a gel (12.5%) with the 4 bands appropriately labelled. This gel pattern compares favorably with other similarly purified (0.6M KCl) microsomal fractions isolated from rabbit hind limb muscles (Martonosi and Beeler, 1983; Campbell, et al., 1980). The identity and function of the remaining protein bands is uncertain. Their presence may indicate only partial purification of the microsomal fraction or, alternatively, some may result, possibly, from a species dependent association with the SR membrane. A particular concern was contamination of the microsomal fraction from phosphorylase b, a 99,000 dalton protein associated with glycogen metabolism. Maclennan (1970) identified that this protein may contribute to ATP hydrolysis and its presence may escape detection in poorly resolved gels since it may form part of the 100,000 dalton band. To this end a 7.5% SDS gel which permits greater resolution was prepared (Plate 4). In Plate 4 purified C and E rat microsomal fractions were compared with an unpurified rabbit fraction from which the presence of contaminating

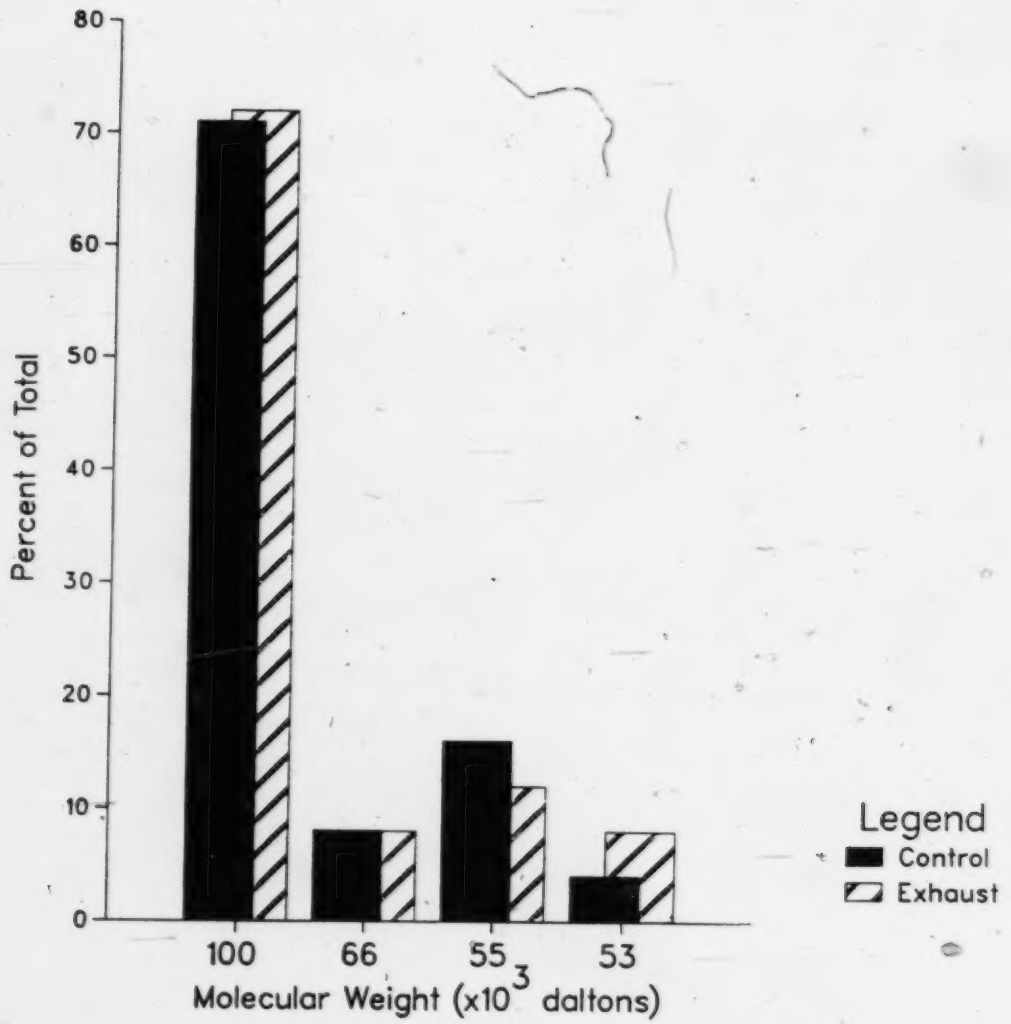

Figure 3.1 Histogram of relative distribution of 100 kDa Ca^{2+}-ATPase, 66 kDa Calsequestrin, 53 kDa Glycoprotein in C and E. The histogram was constructed from densiometric scans of 12.5% SDS gels.

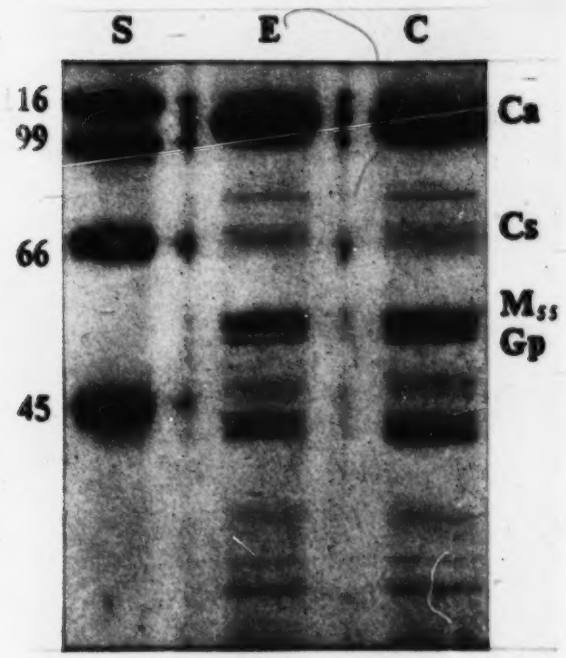

Plate 3. SDS gel (12.5%) of 0.6M KCl purified SR microsomal fraction for E and C. Molecular weight marker proteins (S) are appropriately labelled (x10^3 daltons). In descending size order these are; β-Galactosidase, Phosphorylase, BSA, Ovalbumin. E and C proteins are 100 KDa Ca^{2+}-ATPase (Ca), 66 kDa Calsequestrin (Cs), 55 kDa M_{55} protein (M_{55}), 53kDa Glycoprotein (Gp).

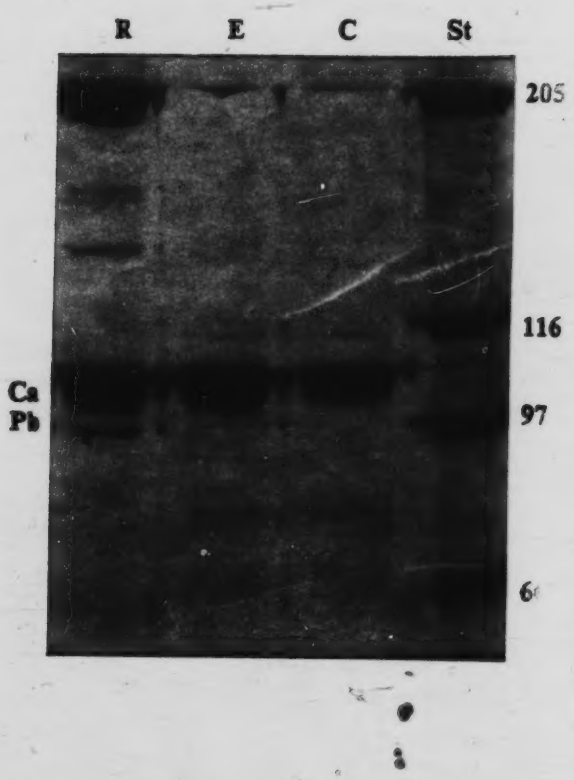

Plate 4. SDS gel (7.5%) of 0.6M KCl purified SR microsomal fraction for R (rabbit hindlimb), E and C proteins. The St well represents marker proteins (x10^3 daltons). In size order these are; Myosin, β-Galactosidase, Phosphorylase, BSA. Ca is the Ca^{2+}-ATPase and Pb is Phosphorylase b in the R fraction.

phosphorylase b protein was originally identified by MacLennan (1970). The relative purity of the rat microsomal fractions can be appreciated by virtue of the absence of phosphorylase b which is present in the rabbit fraction.

The Ca^{2+} transport data expressed as nmoles Ca^{2+}.mg SR protein^{-1} (mean$\pm$SEM) are reported after having subtracted the ATP-independent Ca^{2+} binding values (C=10.64$\pm$1.42; E=11.29$\pm$1.09 ($p<0.05$)). Ca^{2+}-loading (with oxalate) for both C and E tissues increased curvilinearly with time without statistically significant differences ($p>0.05$) between the two groups at any of the 3 time points studied (Figure 3.2). Maximum Ca^{2+}-loading, (at 3 minutes), was 1506$\pm$66 and 1558$\pm$87 nmoles.mg^{-1} for C and E tissue, respectively. The extent of Ca^{2+}-loading is comparable with similar values (1800 nmoles.mg^{-1}) reported by Chu et al. (1983) for fast twitch rabbit back and hindlimb muscle. Although the Ca^{2+}-loading assay employed by Chu et al. (1983) is very similar to that used in this study, the slightly lower peak Ca^{2+}-loading value may be due to species or muscle fibre type differences. Muscles with a greater proportion of fast twitch fibres exhibit greater Ca^{2+} transport capabilities than slow twitch fibres. This is possibly due to higher Ca^{2+}-ATPase/lipid ratios in the SR of fast twitch fibres (Jorgensen et al., 1983).

Unlike the time dependent profile for Ca^{2+}-loading, maximal Ca^{2+}-uptake (without oxalate) appeared to occur within 1 minute beyond the initiation of the reaction (Figure 3.3) Thereafter, Ca^{2+}-uptake reached a plateau which was maintained for both C and E tissue at all 3 time points. The rapid rise to peak Ca^{2+}-uptake (within 1 minute) is a common observation when precipitating anions are excluded from the reaction medium. The attainment of the peak value often occurs well before 1 minute has elapsed. Consequently, determination of true initial rates of Ca^{2+}-uptake are limited by the rapidity with which aliquots can be repeatedly sampled, filtered and then rinsed. In the present study initial rates of Ca^{2+}-uptake cannot be accurately determined from interpolation of the curve since the earliest sampling time point was at 1 minute.

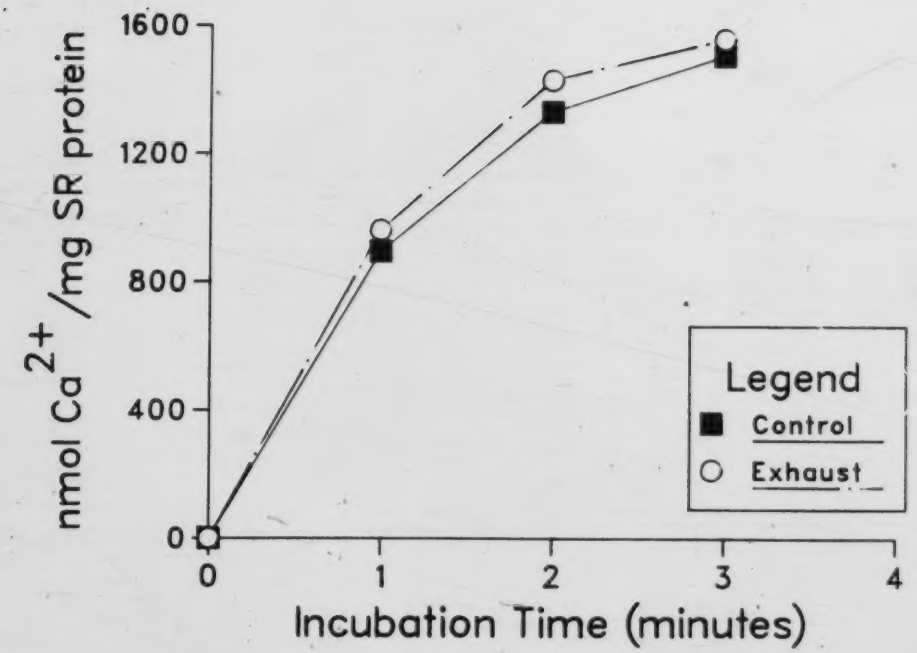

Figure 3.2 Incubation time dependency of Ca^{2+} loading (oxalate supported Ca^{2+} accumulation) for C and E.

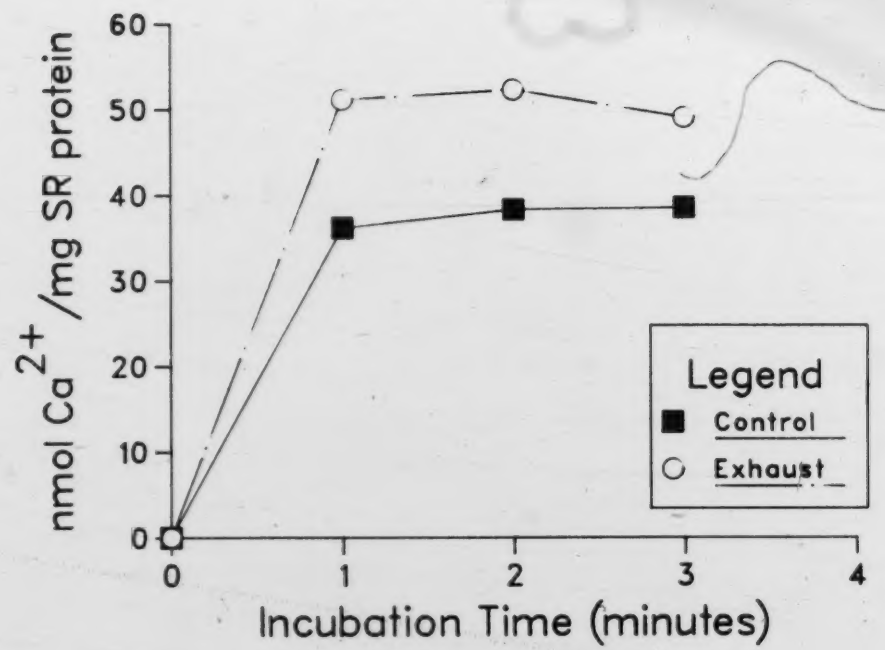

Figure 3.3 Incubation time dependency of Ca^{2+} uptake (no oxalate) for C and E.

The plateau between 1 and 3 minutes (Figure 3.3) indicates the achievement of steady state Ca^{2+}-uptake. During this period the rate of Ca^{2+} influx equals the rate of Ca^{2+} efflux into and out of the vesicle, respectively. From Figure 3.3 it can be seen that significant increases in Ca^{2+}-uptake were observed for E tissue at all time points ($p<0.05$) in comparison to C. At 1 minute incubation C and E values were 36.18 ± 2.5 and 51.15 ± 7.3 nmoles.mg^{-1}, respectively. At 3 minutes the respective values were 38.56 ± 1.2 and 49.07 ± 5.3 nmoles.mg^{-1}. The values obtained are, however, less than those usually reported (70-80 nmoles Ca^{2+}.mg^{-1}) for rabbit fast twitch muscle under similar assay conditions (Chu et al., 1983). This is likely due to the different muscle type used in this study since post hoc experiments with rabbit fast twitch muscle prepared and assayed under identical conditions resulted in Ca^{2+}-uptake values of up to 90 nmoles Ca^{2+}.mg^{-1} (data not shown). In general terms the increase in Ca^{2+}-uptake by E suggests either a) an increase in Ca^{2+} influx, b) a decrease in Ca^{2+} efflux, c) an increase in the storage capacity of the vesicle or, d) a combination of two or more of the foregoing.

Tables 3.1 and 3.2 summarize the mean Ca^{2+} retained by the filter trapped vesicles after rinses with potassium gluconate and KCl (with and without sucrose). The assay conditions (without sucrose) for the accumulation of Ca^{2+} by the vesicles are essentially similar to those employed for Ca^{2+}-uptake since no precipitating anion is present. Subsequent washing of the filters with potassium gluconate does not cause a release of the Ca^{2+} from the filter trapped vesicle. Gluconate is a relatively membrane impermeant anion and will not result in anion induced Ca^{2+} release. Rinsing filters with iso-osmotic KCl does, however, cause a release of Ca^{2+} since the amount of Ca^{2+} retained by the filter is less than that after washing with potassium gluconate. This result supports the reasoning for washing Ca^{2+}-uptake and Ca^{2+}-loading filters with H_2O rather than reaction buffer (without Ca^{2+}).

When vesicles were assayed in a sucrose containing reaction medium the amount of accumulated Ca^{2+} is much higher than when sucrose is absent. Campbell and Shamoo (1980) suggested that sucrose incubation reduces the osmotic gradient against which the Ca^{2+}-ATPase has to work while Ca^{2+} is being accumulated by the vesicle. The Ca^{2+} accumulating energy of

Table 3.1: Summary of Ca^{2+}-release data (mean±sem): Ca^{2+} remaining on filter after washing ($nmol.mg^{-1}$).

Wash solution	Control	Exhaust
K Gluconate	53.28±7.3	60.60±7.5
KCl	22.05±7.3	14.29±4.1
Ca^{2+} released	31.23±3.7	46.31±4.4

the pump may be effectively increased as a consequence. In contrast to the findings of Koshita et al. (1982), rinsing the vesicles with KCl appears to cause a release of Ca^{2+}. Koshita et al. (1982) could not demonstrate anion induced Ca^{2+} release in the presence of sucrose and argued that this manner of Ca^{2+} release (in the absence of sucrose) was an osmotically induced phenomenon. Closer inspection of their data suggests, however, that the microsomal fraction was significantly contaminated with mitochondria since the reported Mg^{2+} stimulated ATPase activity represented 50% of the total $Ca^{2+}+Mg^{2+}$ stimulated ATPase activity. The apparent anion induced Ca^{2+} release reported here is in agreement with similar findings by Campbell and Shamoo (1980), Caswell and Brandt (1981) and Kasai and Miyamoto (1978). Campbell and Shamoo (1980) demonstrated that this manner of release may be associated solely with the "heavy" SR vesicular fraction the origin of which is presumed to be the terminal cisternae. This is consistent with the view that, in vivo, Ca^{2+} release occurs in the cisternal region of the SR rather than throughout the entire reticular membrane (Martonosi, 1984). In the present study, the apparent anion induced Ca^{2+} release is observed for both C and E (Table 3.2). Despite the elevation in both accumulated and released Ca^{2+} for E, these differences were statistically non-significant ($p>0.05$). The large variability within the raw data (appendix B, Table F) may be instrumental in the lack of statistical significance. The reason for this may be the noted inability (despite attempts) to generate a consistent rate of filtration across the filter. The vacuum pump employed did not possess a pressure regulator and was not designed to generate partial vacuums. The desired pressure differential across the filter (10 cm Hg) was created by adjustment of a crude valve device and measured using a simple U-shaped mercury manometer.

Table 3.2: Summary of sucrose Ca^{2+}-release data (mean±sem): Ca^{2+} remaining on filter after washing(nmol.mg^{-1}).

Wash solution	Control	Exhaust
K Gluconate	151.28±10.4	165.30±10.3
KCl	117.40±12.5	109.44±6.4
Ca^{2+} released	33.88±4.3	55.86±9.8

A variable trans-filter flow rate of solvent could be observed as a consequence of the imprecise pressure regulation. Flow rate is a crucial variable is this type of experiment since the Ca^{2+} is released from the vesicle in a non-linear, time dependent manner. The longer the exposure time of the vesicles to the wash media, the greater the amount of released Ca^{2+}. Had the flow rate been more adequately controlled a less variant set of data may have resulted. Further and more precisely controlled experiments are evidently required to investigate the effects of endurance type of exercise upon this manner of Ca^{2+} release.

Figure 3.4 is a plot of Ca^{2+} stimulated-Mg^{2+} dependent ATPase activity versus substrate ($Mg{\cdot}ATP^{2-}$) concentration for both C and E tissues. In both instances a hyperbolic relationship appears to exist between the 2 parameters which is indicative of first-order Michaelis-Menten type kinetics. This is confirmed in Figure 3.5 by the linearity of the subsequent Hoftsee plot. Within the range of Mg·ATP concentrations employed the substrate dependent kinetic profile is an expression of low affinity ATP regulatory site activation (Moller et al., 1980). From Figure 3.4 it appears that the Vmax of ATP hydrolysis is depressed in E tissue. This is consistent with previous reports (Sembrowich and Gollnick, 1977; Belcastro et al., 1981) which suggested that this reflected an ETE induced dysfunction in the Ca^{2+}-ATPase. Since the Ca^{2+}-ATPase is capable of recycling Pi, the suggestion that a drop in net free Pi represents a depression in the catalytic potential of the Ca^{2+}-ATPase may not be entirely valid. Such a case may indicate elevated Pi incorporation into the resynthesis of ATP or in more general terms, it may represent altered regulatory site modulation of Ca^{2+} transport without necessarily implicating depressed catalytic site function. The results of the present study do not address themselves to

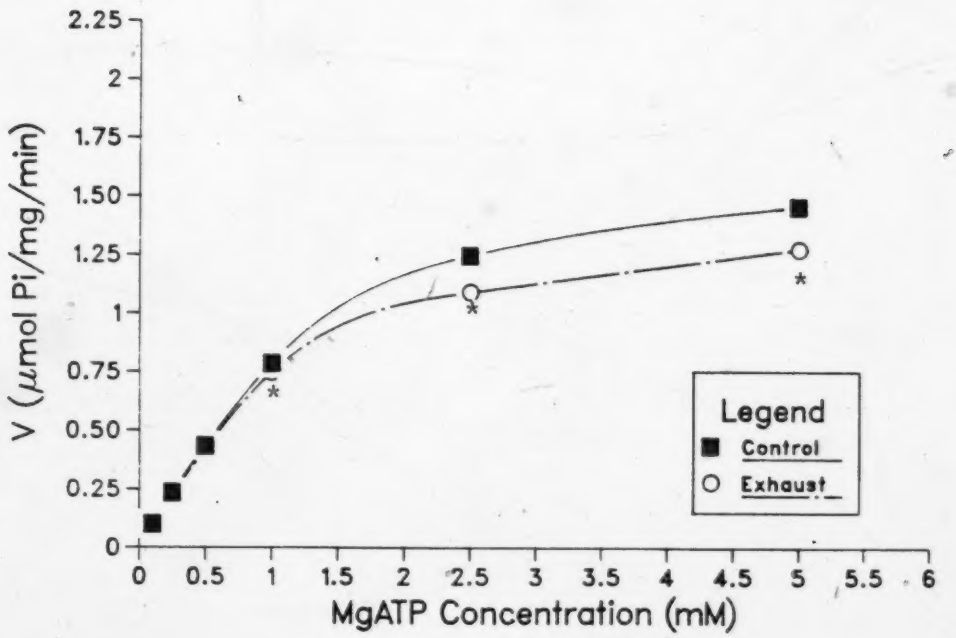

Figure 3.4 Mg·ATP dependency (0-5mM) of Ca^{2+}-ATPase activity in °C and E.

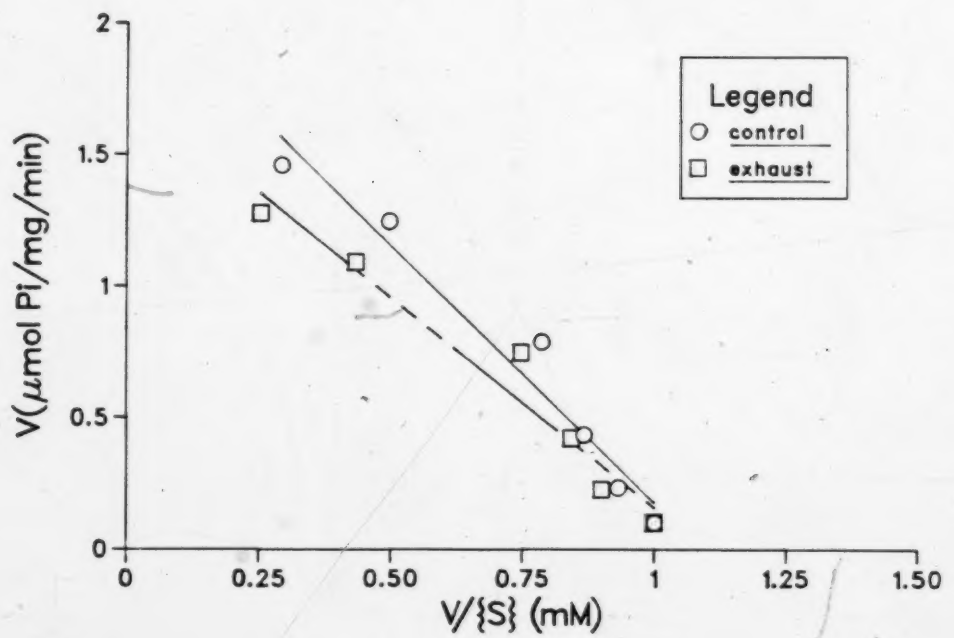

Figure 3.5 Hoftsee plot of Mg·ATP dependency (0-5mM). V= enzyme velocity, [S]= Mg·ATP concentration.

Table 3.3: Derived Km and Vmax values of ATP hydrolysis.

	Control	Exhaust
Vmax (μmol.mg^{-1}.min^{-1})	2.14	1.76
Km (mM)	1.96	1.66

the turnover or catalytic potential of the Ca^{2+}-ATPase since the range of substrate concentrations was insufficiently low. They do however suggest an ETE induced alteration in regulatory site modulation since substrate affinity for the the regulatory site is increased (Figure 3.5; Table 3.3). For E the derived Km value is 1.66mM whereas for C it is 1.76mM ($p<0.05$).

CHAPTER 4

Discussion

In accord with Edwards' (1983) definition of fatigue, the exercised animals employed in the present study fulfilled the criterion for total body exhaustion. That particular muscles were recruited during the activity and may have been instrumental in the etiology of the fatigue process is implied from the extent of intramuscular glycogen depletion (Burke and Edgerton, 1975). No quantitative assessment of muscle glycogen content was made in the present study. On the other hand, comparison of histochemical preparations between control and exhausted gastrocnemius muscle indicates that this muscle was recruited extensively during exercise (Plates 1 and 2)

Figure 3.2 indicates that the exercise protocol employed to fatigue the animals did not significantly alter the Ca^{2+}-loading potential of the gastrocnemius SR fraction (Armstrong and Phelps (1984) showed this muscle fibre composition to be 65% fast glycolytic (FG), 28% fast oxidative glycolytic (FOG) and 7% slow oxidative (SO)). This is in partial contrast to Fitts et al. (1982) who reported, after a swimming protocol of considerably longer duration (7 hours), depressed Ca^{2+}-loading in deep vastus lateralis (63% FG, 36% FOG, 1% SO) and soleus (13% FG, 87% SO) muscles. EDL (56% FG, 42% FOG, 2% SO) and superficial vastus lateralis (97% FG, 3% FOG) muscles, however, displayed no significant alterations in Ca^{2+}-loading.

Several factors may be evoked to explain the observed differences between the present study and that of Fitts et al. (1982). Two of these factors are exercise type and exercise intensity. Cardiovascular responses, for instance, to acute aquatic and treadmill exercise (Flaim et al., 1979) in rats have been shown to differ. Flaim et al. (1979) reported no net change in cardiac output with aquatic exercise whereas this parameter increased two-fold during treadmill exercise. Furthermore, comparisons of skeletal muscle blood flow during treadmill and aquatic

exercise showed the former to be significantly elevated over the latter (up to two-fold difference). The atypical cardiovascular responses reported for aquatic exercise may, to some extent, reflect the reflex bradycardia observed with aquatic submersion (Prosser, 1973). Nevertheless, they also indicate differential involvement of muscles (particularly hindlimb) during the two exercise protocols.

Exercise intensity is one of 3 parameters that describes "involvement" of a muscle during a specific type of exercise. The remaining two are total work and duration of work. With respect to intensity, Parkhouse et al.,(1985) showed that muscle fibre recruitment patterns are dependent upon the intensity of the exercise. Since different muscle fibres have different metabolic properties, exercise intensity will (together with exercise type) likely influence responses at the subcellular level (in this case, possibly, Ca^{2+}-loading). However, quantitative comparison of the total amount and intensity of work performed by the muscle or the animal in both the present study and that of Fitts et al. (1982) is not possible at present, although it is probable that they do differ. The data from the present study, therefore, suggests that Ca^{2+}-loading, in muscles from animals fatigued under the constraints of this protocol, is not affected significantly in relation to controls. This is in accord with the findings of Bonner et al. (1976) who, using a similar exercise protocol, showed no significant differences in Ca^{2+}-loading.

With respect to Ca^{2+}-uptake it is apparent that this parameter is elevated at exhaustion (Fig 3.3). The magnitude of the control values, however, are less than those usually reported (75-100nmoles $Ca^{2+}.mg^{-1}$) for Ca^{2+}-uptake by fragmented SR (Chu et al., 1983). This is surprising in view of the fact that Type II fibres, which have been reported to comprise 93% of the rat gastrocnemius muscle (Armstrong and Phelps, 1984), rapidly accumulate Ca^{2+} owing to the high density of Ca^{2+}-ATPase molecules. A potential source of the relative depression may be the free Ca^{2+} concentration within the reaction medium. Of the 50nmoles.ml^{-1} total Ca^{2+}, approximately 25μM was later determined (using an Orion 900 series Ca^{2+} electrode) to exist as free Ca^{2+}. Maximal Ca^{2+}-uptake is suggested to occur in the presence of 1-10μM free Ca^{2+}

(Weber et al., 1963; Hasselbach, 1979) with apparent inhibition at higher (20-25μM) free Ca^{2+} concentrations (Chiu and Haynes, 1980). Therefore, the Ca^{2+}-uptake values reported in this study may be a consequence of supramaximal (ie. inhibitory) Ca^{2+} activation of Ca^{2+} transport. The 28% elevation in accumulated Ca^{2+} does not initially appear to be accounted for by differential Ca^{2+}-binding since this parameter remains unaltered at exhaustion . However, it has been suggested (Inesi et al., 1980; Pick and Karlish, 1982; Martonosi and Beeler, 1983) that Ca^{2+} binding occurs in two successive steps which precede and follow Mg-ATP binding. Consequently, ATP-independent Ca^{2+}-binding, as assayed in this study, may not have been sentitive to potential ETE induced changes in true Ca^{2+}-binding. On the other hand, truly elevated Ca^{2+}-uptake may have resulted at exhaustion, although whether this increase was represented by an elevated intraluminally bound or free Ca^{2+} compartment is unclear. The exercise protocol may, for example, have potentiated Ca^{2+} binding to the acidic protein Calsequestrin. An additional possibility is that, in fatigued SR, maleate permeability may have increased. Maleate co-transports with Ca^{2+} and clamps this cation intraluminally as an osmotically active complex (Beeler, 1983; Chu et al., 1983). Increased maleate permeability may have stimulated Ca^{2+}-uptake. The Ca^{2+} release data, however, suggests that true increases in Ca^{2+}-uptake occurred. In several different reaction media, a trend towards the same result was obtained.

With respect to the Ca^{2+} release data no significant differences in the amount of released Ca^{2+} from C and E SR were noted. This is substantiated by the lack of interaction between A (wash) and B (group) main effects after 2-way analysis of variance (Appendix C). The dramatic increase in Ca^{2+}-uptake (after potassium gluconate wash) when vesicles were pre-incubated in sucrose (Table 3.4) is consistent with previous reports (Caswell and Brandt, 1981; Mitchell et al., 1984). Mitchell et al. (1984) and Beeler (1983) suggested that sucrose effects a reduction in the transmembranous osmotic gradient which will reduce the chemi-osmotic work of the Ca^{2+}-ATPase; hence elevated Ca^{2+}-uptake. The magnitude of this effect, though, has been suggested to vary with vesicle/sucrose pre-incubation time (Beeler,

1983; Martonosi, 1984). Accordingly, although sucrose incubated vesicles, in the present study, released a proportion of calcium (indicative of non-osmotic release) the true meaning of this result awaits more comprehensive evaluation of sucrose-mediated effects. Additionally, precise comparison of Ca^{2+} release profiles between C and E may be precluded by the evident variability within the Ca^{2+} release data (Appendices B and C). Although anion substitution (chloride for gluconate) consistently releases a proportion of accumulated Ca^{2+}, several factors may compound the variability that is apparent within this particular technique (Nayler and Dressel, 1984; Koshita et al., 1982). Pilot studies conducted prior to this work indicated, for instance, that the amount of Ca^{2+} retained by the filter trapped vesicles, after they were washed with H_2O, was inversely proportional to the filtration vacuum strength. Therefore, although attempts were made to maintain vacuum strength, the vacuum dependent rate of filtration (which appeared to vary considerably) of the wash solutions will partly determine the total amount of Ca^{2+} released since this is dependent upon the exposure time of the trapped vesicles to the wash solution. Consequently, the simultaneous operation of a set of uncontrolled determinants may have yielded a significant within-group variability. Further work is evidently needed to improve the reliability and sensitivity of this technique. This work might include a time dependent analysis of Ca^{2+} release in order to assess both the rate and magnitude of release.

With respect to the kinetic data for ATP hydrolysis it is apparent (Fig 3.4) that the total Pi production is depressed in the fatigued SR. The 18% depression observed in this study is consistent with a similar value (21%) reported by Belcastro et al. (1981). Unlike previous fatigue related studies, though, ATP hydrolysis was monitored in terms of its substrate dependency in the absence of Ca^{2+} precipitating anions. It was, earlier, suggested that depressions in Vmax represented some functional impairment of the Ca^{2+}-ATPase due to fatigue (Sembrowich and Gollnick, 1977). Indirect support for fatigue associated depression in ATP hydrolysis was demonstrated by Dawson et al. (1980). These authors suggested that the maximum rate of ATP hydrolysis (and supposedly Ca^{2+} transport) may be depressed at

exhaustion since ATP potentials were found to be directly proportional to the relaxation time constant. This reasoning, however, presumes that the relaxation time constant, which is depressed at exhaustion, is primarily a function of the rate of Ca^{2+} sequestration rather than the rate of crossbridge cycling. Furthermore, the efficiency of the Ca^{2+}-ATPase and thus the energy coupling of Ca^{2+} transport is not, solely, a function of ATP potentials but also determined by the relative concentrations of the bound and free forms of Ca^{2+} associated with the SR (Hasselbach and Waas, 1982).

In vitro, the issue of ATP potentials or energy charge may not be a limiting factor with respect to ATP hydrolysis and Ca^{2+}-transport since fatigue induced changes can be observed in the presence of optimal (5mM Mg·ATP) substrate concentrations. During the 10 minute reaction period only 10-15% of the total Mg·ATP is consumed for Ca^{2+} transport. Since substrate concentration is far from limiting then it may be feasible to assume that the energy state, in vitro, does not differ between control and fatigued tissue. Consequently, if the foregoing is correctly assumed, then the altered kinetic behavior of the Ca^{2+}-ATPase as observed in this and other studies is presumably due to alterations in (a) some fundamental characteristic of the Ca^{2+}-ATPase, and/or (b) some other regulatory feature of the Ca^{2+}-ATPase. With respect to the former possibility, a depressed Ca^{2+}-ATPase population in relation to total protein can be eliminated since SDS-PAGE gel patterns do not differ between control and fatigue (Fig 3.1). Inspection of Figure 3.5 and Table 3.3 reveals, however, that, despite a reduced Vmax, the enzyme substrate affinity is increased ($p<0.05$). This implies that exhaustion or ETE may indirectly modify the low affinity (regulatory) substrate binding site in order to accomodate the reduced intracellular energy state in vivo. The modifying agent and the mechanism of this change are both uncertain at present. However, the altered ionic environment, in vivo, (particularly Ca^{2+} and protons) associated with exhaustion (Gonzales-Serratos et al., 1978) may be instrumental in this effect.

The elevated Ca^{2+}-uptake in the face of a decreased Vmax for ATP hydrolysis at exhaustion may represent a reduction in the energy cost of Ca^{2+} transport. This apparent

increase in the efficiency of Ca^{2+} transport may in turn be associated with the apparent increase in substrate affinity of the Ca^{2+}-ATPase. The energy of ATP hydrolysis is expended in translocating Ca^{2+} from a binding site of high affinity to a site of low affinity (Hasselbach, 1979). Consequently, a decreased energy cost may reflect a reduction in the thermodynamic gradient that exists between the energy states of the two Ca^{2+} binding sites. Since Ca^{2+} binding to the external high affinity sites was unaltered in this study, low affinity site binding of Ca^{2+} may have been potentiated. Accordingly, modification of the Ca^{2+} binding site affinity has been implicated in determining the variable stoichiometry of Ca^{2+}/ATP ratios (Tanford, 1983). Berman (1982) and Meltzer and Berman (1984), alternatively, have suggested that variable stoichiometry might be determined by an altered probability of Ca^{2+} pump mediated Ca^{2+} fluxes in the reverse and forward directions. In the present study a forward running of the Ca^{2+}-ATPase during the initial phase of Ca^{2+}-influx may be favoured in the fatigued SR due to product (ATP) inhibition of the reverse direction. This may be created, possibly, through an increased affinity of the Ca^{2+}-ATPase for Mg·ATP. This explanation applies, however, only if the stoichiometry of the Ca^{2+}-ATPase actually changes. This cannot be assessed from the present data since initial rates of Ca^{2+} influx, enzyme/phosphate complex formation and Pi turnover were not determined. Alternatively, apparent or net stoichiometric changes may have occurred if the membrane was less "leaky" to Ca^{2+} in the fatigued SR fraction.

In another light, it is possible that the increased Ca^{2+}-uptake represents an increase in the maximal concentrating ability of the SR through altered substrate affinity. It may be possible that, during the initial stages of Ca^{2+}-uptake, Ca^{2+} influx is accelerated through an increase in the rate of return of the luminally oriented translocator to a cytoplasmically oriented conformation (this stage is rate limiting and is accelerated by increases in substrate concentration or substrate affinity). Later, approaching steady state, the rate of Ca^{2+} exchange between the internal and external Ca^{2+} may become more prominent. This may be accompanied by a net reduction in the total amount of ATP hydrolysed since the rate of Pi exchange between ATP and ADP and coincident partial reversal of the Ca^{2+} pump would be stimulated in the

presence of increasing ADP and luminal Ca^{2+} concentrations. Thus, the effective result may be a faster cycling of Ca^{2+} around the Ca^{2+}-ATPase at steady state Ca-uptake. This may reflect a more rapid regulation of the local Ca^{2+} environment by the enzyme, in vivo, in an attempt to overcome a depressed energy state. The foregoing hypotheses, however, obviously require further investigation since changes in other factors such as membrane leakiness, lipid/protein ratios, and vesicle volume may be operating.

The changes observed in the fatigued SR tissue indicate that this organelle may have undergone some positive adaptation during the course of this exercise protocol. Rather than some intrinsic malfunction of the SR, it is suggested that Ca^{2+}-ATPase functioning may have been potentiated in an attempt to maximize the Ca^{2+} concentrating ability in the face of (a) depressed ATP levels (Parkhouse et al., 1985) (b) an imbalanced local Ca^{2+} environment and (c) an altered ionic environment. That adaptation rather than maladaptation occurs is supported by the observation (Gilchrist et al., 1984) that ATP hydrolysis is reduced within the first two minutes of a similar ETE protocol. This depression continued and remained constant between 15 minutes into exercise and exhaustion. The altered SR kinetic parameters, in vitro, therefore, may be more a function of exercise, per se, rather than solely related to events associated with exhaustion. This does not preclude a role for the SR at exhaustion, however, since altered in vivo function may not necessarily be preserved in vitro. Rates of SR Ca^{2+} uptake and release may well be depressed at exhaustion due to a potentially unfavorable thermodynamic environment for which further compensation by the Ca^{2+}-ATPase is no longer possible.

CHAPTER 5

Summary, Conclusions and Recommendations

Summary

The present study attempted to re-evaluate the effects of ETE upon selected Ca^{2+} transport parameters of gastrocnemius muscle (Ca^{2+}-uptake, Ca^{2+}-loading, ATP hydrolysis and anion substitution induced-Ca^{2+} release) at the point of whole body exhaustion in female rats. As a result of the running protocol employed, the following observations were made:

1. ETE had no significant effect upon Ca^{2+}-loading in terms of both initial rate and extent.
2. Ca^{2+}-uptake, on the other hand, was elevated (28%) as a result of exhaustive ETE.
3. Although the Vmax of ATP hydroysis was depressed, the substrate ($Mg{\cdot}ATP^{2-}$) affinity for the Ca^{2+}-ATPase increased as a result of the exhaustive exercise.
4. No significant effects of exhaustive ETE upon the Ca^{2+}-release protocol employed were noted.
5. These changes could not be ascribed to alterations in the protein profile of the SR fraction.

Conclusion

In light of the foregoing it was hypothesised that the changes observed may reflect;

1. decreased energy cost of unidirectional Ca^{2+} influx through altered Ca^{2+}/ATP stoichiometry and/or
2. an increase in the rate of cycling of Ca^{2+} around the Ca^{2+}-ATPase: this may establish a faster control of Ca^{2+} flux.

It was suggested that during exhaustive ETE the Ca^{2+}-ATPase may undergo positive adaptation in order to accommodate an altered ionic environment and reduced energy state, in

vivo. These changes may be induced by the intracellular environment itself (eg. Ca^{2+} overload; markedly decreased by pH) possibly in a feedback dependent manner.

Recommendations

The following issues are recommended for future study with SR and ETE fatigue in order to establish a more comprehensive role for the SR.

1. Ca^{2+} dependency of Ca^{2+} transport parameters and ATP hydrolysis.
2. Study of the stoichiometry of Ca^{2+} transport through examination of initial rates of Ca^{2+} uptake, Ca^{2+} loading and ATP hydrolysis.
3. The role of membrane permeability to Ca^{2+}; the role of lipid/protein ratios; the role of vesicle volume; and the role of osmotic influences upon Ca^{2+} transport.
4. The role of Ca^{2+} binding proteins and the influence of potential alterations in Ca^{2+} binding properties.
5. The effects of intensity, duration and workload upon the foregoing parameters. Although the changes observed in their study were noted at exhaustion they may not necessarily reflect events at exhaustion. It will be necessary in future, therefore, to examine the time course of these changes in order to identify and discriminate a relationship between events associated with exercise and events causally related to exhaustion.

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Appendix A - Review of Related Literature

Morphology of SR

The SR of skeletal muscle is characterized by the presence of two morphologically, and functionally distinct regions (Peachey and Franzini-Armstrong, 1983). These regions are i) the junctional SR (jSR) which is found in close proximity to the transverse tubular system (T-tubules) and, ii) the free SR (fSR) which forms the longitudinal and fenestrated regions of the reticulum. Although the detailed structure may depend upon the muscle and animal type (Peachey, 1965), this classification is consistent for all muscles including the myocardium (Jewett et al., 1971).

The freeze-fractured fSR is characterized by a dense population of 85-90 angstrom particles on the cytoplasmic fracture face. Furthermore, the membrane is particularly asymmetric since its luminal face shows hardly discernable indentations and very few particles (Deamer and Baskin, 1969; MacLennan et al., 1971; Franzini-Armstrong, 1975, 1980). The hypothesis that the intramembraneous particles represent the Ca^{2+} pump is supported by the correlation between the 85 angstrom particle density and i) the rate of Ca^{2+} influx, ii) the Ca^{2+} stimulated ATPase activity, iii) the speed of contraction of various muscles (Baskin, 1971; Rayns et al., 1975; Devine and Rayns, 1975; Martonosi, 1980). In fast twitch muscle the density of the 85 angstrom particles is approximately 3,000-5,000 per μm^2 across the entire surface of the fSR (Franzini-Armstrong, 1975). In slow twitch muscle the particle density is approximately half that value as determined by indirect immunoferritin labeling of ultrathin frozen sections (Jorgensen et al., 1983).

The Ca^{2+} transport protein is transmembraneous (Hidalgo and Ikemoto, 1977) and protrudes from the cytoplasmic face into the aqueous phase to form 40 angstrom particles that are identified after negative (Inesi and Scales, 1974) and tannic acid (Saito et al., 1978) staining. The density is approximately 4-6 times that of the 85 angstrom particles (Martonosi, 1984). In order to account for the discrepant distribution and densities of the cytoplasmic and

intramembraneous particles. Vanderkooi et al. (1977), using fluorescence energy transfer in reconstituted vesicles, suggested that the 85 angstrom particles represent clusters of oligomers of ATPase molecules. Support for this concept is presented through the observation of 200,000 and 400,000 dalton oligomers after sedimentation in detergents (Le Maire et al., 1976) and chemical crosslinking (Chyn and Martonosi, 1977).

The morphological transition from the fSR to the jSR or terminal cisternae is particularly pronounced. The 85 angstrom particles of the fSR are absent in this region with the appearance of larger and less densely packed particles observed on the cytoplasmic leaflet after freeze fracture (Martonosi, 1968) and immunoferritin labelling (Jorgensen et al., 1983). The packing density of the jSR particles is much less than the fSR particles and Martonosi (1984) suggested that the sharp transition between the two regions, possibly, create a barrier to free diffusion.

The close apposition of the jSR and the T-tubles results in the formation of triads. The flattened surfaces of 2 cisternal sacs of the jSR flank the junctional surface of a T-tubule. Occasionally, in the myocardium and the muscles of invertebrates, only one element of the SR forms a junction with a T-tubule (diad) (Martonosi and Beeler, 1983). The junctional gap between the T-tubule and the jSR is approximately 100-200 angstroms and is characterized by the presence of dense projections or feet which connect the cytoplasmic leaflets of the two membranous systems (Franzini-Armstrong, 1980). The feet are periodically dispersed at intervals of 300 angstroms and are arranged in rows of 2 or 3 on both sides of the T tubule (Jewett et al., 1971). The feet are considered to be associated with the jSR rather than the T-tubule since they remain associated with the jSR upon fragmentation and fractionation of the reticular membrane (Campbell et al., 1980).

Additional junction spanning structures have also been identified after tannic acid staining (Somlyo, 1979). These structures or membranous bridges create continuity between the cytoplasmic leaflets of the triad membrane and are spaced similarly to, and probably run centrally through, the junctional feet (Franzini-Armstrong, 1980). Somlyo (1979) has

proposed that these bridges may be phosopholipid structures although the suggestion was based soley on their staining properties. The precise role for both sets of junction spanning structures has not been identified. Since the luminal leaflets of the triadic membranes appeared separate, Martonosi (1984) argued against direct electrical coupling between the T-tubules and the SR. Somlyo (1979), however, argued that the bridges were compatible with charge movement theories of Ca^{2+} release (Schnieder and Chandler, 1973; Adrian and Almers, 1976). In principle, their presence could account for the time interval between depolarization and Ca^{2+}-release.

Protein Composition of SR

Upon mechanical disruption of the muscle cell in salt or sucrose containing solutions the SR membrane becomes fragmented. The SR membrane fragments then form scaled spherical vesicles (80-100nm mean diameter) which can be harvested in high yields. The formation of such vesicles from irregular fragments is, in itself, a remarkable property of this membrane since, although this process is thermodynamically essential for the lipid fraction, it demonstrates the high degree of mobility of the other components (Hasselbach, 1979). Meissner (1975) reported that 90% of the protein in the light fraction was the Ca^{2+}-ATPase protein whereas the latter constituted 55-60% of the total protein in the heavy fraction. An additional component found almost exclusively within the heavy fraction is Calsequestrin, an acidic protein that possesses Ca^{2+} binding properties (Maclennan and Wong, 1971). Meissner (1975) also noted the presence of matrix material within the terminal cisternae and the heavy vesicular fraction. He suggested that the matrix was formed from Calsequestrin and that the heavy vesicular fraction was derived from the terminal cisternae. This suggestion received additonal support from the later work of Lau et al. (1977) who reported electron dense material within the heavy SR which they presumed to be Calsequestrin.

The SR proteins comprise, approximately, 65% of the microsomal dry weight (Inesi, 1981) of which 50-80% is the 100,000 dalton Ca^{2+}-ATPase (Ohnoki and Martonosi, 1980). Besides Calsequestrin (Mr=44,000), a High Affinity Calcuim Binding Protein (Mr=55,000)

(Ostwald and Maclennan, 1974), a 12 kDa Proteolipid (Maclennan et al., 1972) and a 53 kDa Glycoprotein (Michilak et al., 1980) have also been identified. The SR protein profile, as revealed by SDS-PAGE, is relatively simple after high salt extraction of the surface proteins normally associated with an endoplasmic reticulum. A first approach to the identity of the 100,000 dalton protein was established by labelling the membrane with ^{14}C N-ethyl-maleimide, a thiol reagent that specifically blocks Ca^{2+} transport (Hasselbach, 1966). The label was eluted from a Sepharose 4B column at a volume normally associated with molecular weights of approximately 100,000. The identity of the 100,000 molecular weight protein was later confirmed (Martonosi, 1969) through covalent attachment of ATP, labelled at the terminal phosphate with ^{32}P. The Ca^{2+}-ATPase can be purified and solubilised in low yields (Maclennan, 1970) through the use of deoxycholate as a detergent. The level of detergent is crucial, however, since excesses can render the Ca^{2+}-ATPase inactive through conformational perturbations.

Ca^{2+}-ATPase Structure

Exposure of the SR to mild tryptic digest can degrade the Ca^{2+}-ATPase into two major fragments: a 55,000 dalton (A) fragment and; a 45,000 dalton (B) fragment (Thorley-Lawson and Green, 1973; Inesi and Scales, 1974). Despite this digestion the Ca^{2+}-ATPase retained its hydrolytic potential and both fragments remained associated with the membrane. The incorporation of ^{32}P into A indicated that this fragment contains the site of ATP hydrolysis (Stewart et al., 1976). Extended digestion of fragment A resulted in 2 smaller subfragments: a 33,000 dalton (A_1) fragment and; a 22,000 dalton (A_2) fragment. This subsequent digest did not, however, affect fragment B. Furthermore, ^{32}P incorporation into the Ca^{2+}-ATPase prior to the extended digest resulted in the label recovery from fragment A_1. Stewart et al. (1976) have sequenced fragment A_1 and indentified the active site as a phosphoaspartyl residue. Consequently, fragment A_1 was suggested to have a cytoplasmic orientation while fragments A_2 and B were mostly buried within the membrane. Allen et al., (1980) deduced that the alignment of the fragments was A_2-A_1-B with A_1 possessing the N-Ac-Met terminal sequence. This alignment was confirmed by Klip et al. (1980) who proposed that A_2 and B fragments

possessed atleast one transmembrane passage each and A_1 possessed atleast two. At that time, however, 200 membrane associated amino acids had yet to be sequenced. Consequently, Klip et al. (1980) suggested that the Ca^{2+}-ATPase may make up to 8 transmembrane passages in all.

The precise mechanism of Ca^{2+} transport remains obscure (Tong, 1980) although the NH_2 terminal region of fragment A_2 is suggested to contain ionophoretic activity (Shamoo et al., 1976). Observations regarding the ionophoretic activity of the A_2 fragment do not clearly implicate a physiological role, however. Physiologically, the permeability selectivity by the Ca^{2+}-ATPase for Ca^{2+} and Mg^{2+} is marked although addition of the A_2 and B fragments, in vitro, resulted in relatively non-selective permeability for these ions (Shamoo and Maclennan, 1974). Furthermore, increases in ionic current across the membrane upon addition of the A_2 fragment were pronounced only at millimolar concentrations of Ca^{2+} and minimal at physiological levels (pCa8-pCa5). More recently, the entire amino acid sequence of rabbit muscle SR Ca^{2+}-ATPase was deduced through cloning of complementary DNA which encoded two forms of this enzyme (MacLennan et al., 1985). Knowledge of the primary structure led to a prediction of the secondary and tertiary structure in which the protein was proposed to possess 3 cytoplasmic domains linked to 10 transmembrane helices via a penta-helical stalk. One domain was identified as the nucleotide binding site which is separate from the phosphoaspartyl site located upon an adjoining domain. The third cytoplasmic domain was suggested to be a transduction domain as cleavage at the second tryptic site (located within this domain) uncouples Ca^{2+} transport from ATPase activity which remains intact. The penta-helical stalk was suggested to possess the high affinity Ca^{2+} binding site by virtue of the amphipathic α-helical segments being enriched with glutamic acids residues. Very little of the protein is located upon the luminal face of the membrane. However, on the loop between the first and second transmembrane helices a group of 4 glutamic acid residues was suggested by MacLennan et al. (1985) to be the low affinity Ca^{2+} binding site.

Calsequestrin Structure

Calsequestrin, one of the major accessorial proteins of the SR, is an acidic glycoprotein (Maclennan and Campbell, 1979) that has Ca^{2+} binding properties (Kd=1mM at physiological ionic strength). It is an extrinsic membrane protein localized preferentially within the terminal cisternae upon the luminal surface (Hasselbach et al., 1975; Hidalgo and Ikemoto, 1977). Calsequestrin has an apparent molecular weight of 44,000 when identified upon Weber and Osborne gels (Weber and Osborne, 1969) although Laemmli gels (Laemmli, 1970) indicate a molecular weight as high as 63,000. Martonosi and Beeler (1983) noted that the apparent discrepancy may be due to the pH differences between the two gel systems. This was later confirmed by Cozens and Reithmeier (1984) who extensively characterised the physiochemical properties of Calsequestrin. The true molecular weight of Calsequestrin was found to be approximately 40,000. Cozens and Reithmeier (1984) reported that Calsequestrin is highly asymmetric with an extended structure created by the electrostatic repulsion amongst neighbouring negative charges. However, the higher Ca^{2+} accumulating ability of the "light" vesicles over the "heavy" vesicles (Meissner, 1975; Campbell et al., 1980) does not support the concept of Ca^{2+}-binding by Calsequestrin as being physiologically important. Consequently, the role of Calsequestrin is presently uncertain since other Ca^{2+} binding components may be involved in Ca^{2+} accumulation in vivo (Vale and Carvalho, 1975; Vale et al., 1976).

High Affinity Ca^{2+} Binding Protien (HABP) Structure

HABP is the second SR Ca^{2+} binding protein with an apparent molecular weight of 55,000. It is an acidic protein that is localized upon the luminal surface of the SR membrane (Maclennan et al., 1972; Stewart and Maclennan, 1974). HABP binds Ca^{2+} with lower capacity but higher affinity (Kd=3μm) than Calsequestrin. When native vesicles are prepared and separated into "light" and "heavy" fractions (Meissner, 1975) HABP is localised within the heavy fraction (derived from the terminal cisternae). Subsequent French press treatment of the cisternal fraction produces a sub-population of light and heavy vesicles (Lau et al., 1977;

Michalak et al.; 1980). Lau et al. (1977) showed that the lighter fraction was relatively enriched with HABP (in relation to Calsequestrin). Michalak et al. (1980) suggested that HABP is part of the sarcotubular membrane system and may form a communication link between the transverse tubules and the terminal cisternae. However, the physiological function of HABP is obscure. It's Ca^{2+} binding properties resemble that of regulatory proteins such as Troponin-C. On the other hand, it is located upon the luminal face of the SR network where Ca^{2+} concentrations are sufficiently high (10mM) to preclude a regulatory role for HABP.

53,000-dalton Glycoprotein Structure

Using Laemmli (Laemmli, 1970) gels to separate and identify possible contaminants within the 55,000-dalton band of Weber and Osborne gels (Weber and Osborne, 1969), Michalak et al. (1980) discovered a glycoprotein with a molecular weight of 53,000. Unlike the glycoprotein of the $Na^+ + K^+$-ATPase, however, the 53 kDa protein of purified SR did not form a tight association with the Ca^{2+}-ATPase. The 53 kDa glycoprotein was later purified and shown to be transmembranous owing to it's reactivity with concanavalin A in leaky vesicles and cycloheptaamylose-fluorescamine complex in sealed vesicles (Campbell and MacLennan, 1981). Subsequent photo-affinity labelling studies (Campbell and MacLennan, 1983) with 8-N_3-[α-^{32}P]ATP have shown that the 53 kDa protein possesses high affinity sites for ATP. The function of this glycoprotein is obscure although Campbell and MacLennan (1983) suggested that it may be a membrane-bound protein kinase.

Lipid Composition of SR

The SR contains approximatley 0.6mg lipid/mg protein such that membrane lipids constitute 35% of the membrane matrix (Waku et al., 1971). Of the total lipid, 80% is in the form of phospholipids (PL) on a molar basis. This corresponds to an association of 90-100mol PL/mol Ca^{2+}-ATPase (Tada et al., 1978). Phosphatidylcholine (PC) is the most abundant PL (65-73%), although the presence of the phosphatidylethanolamine (PE)(12-19%) is required for maximal Ca^{2+}-transport (Knowles and Racker, 1975). The remaining classes of PL are

phosphatidylinositol (PI)(9%), phosphatidylserine (PS)(2%), sphingomyelin (4%) and, cardiolipin (0.1-0.3%). The remaining lipid is neutral of which 95% is cholesterol. The lipid content and lipid/ protein ratio do, however, vary depending upon the species of animal and muscle type.

As with many different biological membranes, an asymmetric bilayer distributition of PLs is apparent within the SR membrane. Nuclear Magnetic Resonance (NMR) studies (Dekruijff et al., 1979), using a chemical shift reagent (Dy^{3+}), indicate that 60% of PC is on the luminal leaflet of the SR. PE and PS, however, appears to be located, almost exclusively, upon the cytoplasmic leaflet (Hidalgo and Ikemoto, 1977). Much of the information regarding bilayer distribution of membrane PLs has ,however, been obtained from experiments conducted upon plasma membranes. Furthermore, it has been suggested (Bennett et al., 1980) that many of the techniques may, themselves, perturb the membranes to the extent that experimental artifacts are a likely result. Consequently, the role of PL bilayer asymmetry in biological systems is uncertain (Rothman and Lenard, 1977; Zwaal et al., 1977). This is especially true of intracellular membranes (e.g. SR and Endoplasmic Reticulum) since it is not known whether asymmetry represents a state preserved from biosynthesis (Jelsema and Morre, 1978) or determined by specific membrane bound enzymes (Bretscher, 1973).

Despite the above, the Ca^{2+}-ATPase appears to be critically dependent upon the surrounding lipid environment for enzyme activity (Bennett et al., 1980). Although a 90-100mol PL/mol Ca^{2+}-ATPase ratio exists in vivo, a 30mol PL/mol Ca^{2+}-ATPase stoichiometry was found to maintain ATPase activity after cholate extraction of PLs (Hesketh et al., 1976; Knowles et al., 1976). Upon further removal of PLs, virtual inactivation of ATPase activity resulted below a molar ratio of 15 PL/ATPase (Warren et al., 1974). This loss of activity is not irreversible, however, since restoration of almost full activity can be achieved by the readdition of various PLs (Melgunov and Akimova, 1980). In some cases non-ionic detergents can also effectively substitute PLs (Dean and Tanford, 1977,1978).

Experiments with reconstituted vesicles indicate acyl chain specificity for optimal ATPase functioning (Warren et al., 1974). A dioleoyl PC (DOPC)-ATPase complex was found to be most like the native vesicle with respect to ATPase activity and Ca^{2+} accumulation. Although increasing carbon chain length from 14 to 18 results in a general increase in ATPase activity, the addition of unsaturated double bonds creates a dramatic increase in enzyme activity at 37°C (Bennett et al., 1980). At temperatures above the phase transition temperature, however, reconstituted PL-ATPase complexes displayed ATPase activities comparable to the native SR vesicle. This supports the contention (McElhaney, 1983) that the nature of the lipid polar environment is of little consequence to enzyme functioning as long as the immediate environment exists in a fully fluid, liquid-crystalline state. The temperature dependence of enzyme function in enzyme-lipid complexes, above the phase transition temperature of the lipid, becomes, solely, a property of the enzyme. The apparent requirement for unsaturated long chain fatty acids (e.g.18:1 cis), and hence a fluid lipid matrix at physiological temperatures, appears to be related to the need of the Ca^{2+}-ATPase to undergo conformational changes during Ca^{2+}-transport (Dupont and Leigh, 1978). Hidalgo et al. (1978) and Nakamura et al. (1976) showed that a gel state lipid environment reduces the rate of phosphoenzyme dephosphorylation since this kinetic step is dependent upon Ca^{2+} translocation across the membrane (Ikemoto, 1976). On the other hand, phosphoenzyme formation, which precedes Ca^{2+} translocation (Makinose, 1973), is affected to a much lesser degree.

A question of considerable debate concerns the existence and functional significance of a putative "lipid annulus" surrounding the Ca^{2+}-ATPase . Jost et al. (1973), working with cytochrome oxidase and lipid spin labels, identified the presence of a single layer of protein-immobilized PLs which appeared essential for enzyme function. This work was extended to an examination of the SR (Hesketh et al., 1976) and similar conclusions were drawn. Hesketh et al. (1976), using 5-doxyl-stearate and TEMPO probes, reported anomalous enzyme activity below the DPPC transition temperature and suggested that this reflected a lack of normal phase transition in the annular lipid. They proposed that this boundary lipid

buffered the Ca^{2+}-ATPase against the effect of bulk lipid phase transitions. More recently, Lentz et al. (1982), using diphenylhexatriene (DPH) fluorescence anisotropy, supported the contention that a boundary lipid exists. However, in order to account for the enhanced disorder in the annular lipid (rather than relative immobility), as reported by deuterium Electron Spin Resonance (ESR), Lentz et al. (1982) proposed, in addition to the boundary lipid, the presence of a second lipid layer with disrupted packing order. The presence of an immobilized lipid component of reconstituted SR vesicles was also found by McIntyre et al. (1982) using 5-doxyl and 16-doxyl stearate probes and a spectral subtraction technique of Electron Paramagnetic Resonance(EPR) analysis. However, they cautioned the interpretation of their data since the derived spectra are calculated on an assumed model for "immobilized" PL.

A major problem of ESR and EPR spectral analysis is that the probe of choice, to some extent, perturbs the bilayer and may report on the anisotropic motions of itself rather than any fundamental property of the lipid phase. NMR has been applied to the study of biological membranes with the advantage that membrane perturbation does not result. Albert et al. (1981), Fleischer et al. (1981), and McLaughlin et al. (1981) showed with both ^{31}P-NMR and deuterium-NMR that the protein component induces some perturbation of the lipid environment. However, Albert et al. (1981) using native SR vesicles suggested that the observed immobilization was not due to the Ca^{2+}-ATPase, since increasing salt abolished the immobilization. They ascribed the immobilization to presence of the peripheral proteins since this effect was not observed in their absence.

SR Ca^{2+}-transport

Recognition of Substrates

Transmembranous ion transport by a carrier protein is characterized in terms of recognition, translocation and release of ions (Pardee, 1968). Recognition with respect to active Ca^{2+} transport by the Ca^{2+}-ATPase occurs with the binding of both the translocated Ca^{2+} ion and the Mg·ATP to specific sites located upon the cytoplasmic face of the enzyme (Weber et

al., 1966). Ca^{2+} binding, which has been studied with native and reconstituted vesicles and purified Ca^{2+}-ATPase, occurs with high affinity in the absence of ATP (Meissner, 1973; Ikemoto, 1974; Chiu and Hayes, 1977). Three classes of Ca^{2+} binding sites have been identified in the purified enzyme (Ikemoto, 1975). At 0°C all three binding sites (α, β, and γ classes) were expressed with association constants (k) and number of sites (n) per class as follows: α sites ($k=3x10^6M^{-1}$, $n=1$); β sites ($k=5x10^4M^{-1}$, $n=1$); γ sites ($k=10^3M^{-1}$, $n=3$). Above 22°C, however, the number of α sites increased to 2 with suppression of β site binding and maintainance of low affinity of γ site binding . This result is supported by the finding (Sumida and Tonomura, 1974) that the degree of Ca^{2+} binding is diminished with decreasing temperature. Inesi et al. (1980) showed that Ca^{2+} binding to the high affinity sites of the Ca^{2+}-ATPase is cooperative with a derived Hill coefficient of 1.82 and an apparent affinity constant (Kapp) of $2.3x10^6M^{-1}$. Previous reports (Meissner, 1973; Ikemoto, 1976) failed to observe the cooperativity since, as Martonosi and Beeler (1983) suggested, the binding studies were not extended to very low ($pCa<7$) Ca^{2+} concentrations.

Ca^{2+} binding to the external high affinity sites of the Ca^{2+}-ATPase has been demonstrated to induce conformational changes in the protein (Pick and Karlish, 1980). The Ca^{2+} induced changes were monitored through increases in intrinsic tryptophan fluorescence (Dupont, 1976), through fluorescence quenching of bound fluorescein (Pick and Karlish, 1980) and through changes in the ultraviolet spectrum of the solubilised protein (Nakamura, 1977). The conformational changes associated with Ca^{2+} binding are relatively slow (Rauch et al., 1978; Hasselbach et al., 1981) and this transition is consequently considered to be one of the rate limiting steps of the catalytic cycle (Dupont, 1977; Pick and Karlish, 1982). Rate limiting Ca^{2+} binding, however, is not implied from the above studies (DeMeis and Vianna, 1979) since the isomerisation rate cannot account for maximal Ca^{2+} binding within 3 milliseconds (Chiu and Haynes, 1977).

Mg·ATP is the true substrate for the exergonic translocation of Ca^{2+} (Vianna, 1975). ATP binds to the transport enzyme with high affinity (Meissner,1973) only as a nucleotide-Mg

complex (Hasselbach et al. 1981). Hasselbach et al. (1981) suggested that Mg^{2+} functions both as an ionic cofactor and a charge compensator for the polyphosphate residues of the unliganded nucleotide substrate. The resulting Mg-ATPase-enzyme complex is formed by random binding of the cofactor and substrate to the translocator. Commonly, Ca^{2+} and Mg·ATP bind in a molar ratio of 2 as determined from Hill plot coefficients under optimal conditions (Tada et al., 1978). This stoichiometry is confirmed by the finding that 8-9nmol Ca^{2+} bind to the external affinity sites per mg of Ca^{2+}-ATPase whereas 4-4.5 nmol phosphoenzyme (EP) is formed (Inesi et al., 1982). Ca^{2+} and Mg·ATP binding to the enzyme was thought to occur randomly as implied from the apparent lack of allosteric effect of the two binding sites (Vianna, 1975). More recent studies, however, do not support this view. Chiu and Haynes (1980) found that Ca^{2+} occupation of the high affinity sites prior to substrate binding is crucial to the functional integrity of the Ca^{2+} uptake system. Sumida et al. (1978) suggested that Ca^{2+} binding induces conformational changes in the enzyme which is, subsequently, more conducive to active transport. Furthermore, Pick and Karlish (1982) demonstrated that the binding of only one Ca^{2+} ion is required for the formation of the influx stabilized form of the enzyme. Since the rate of ATP hydrolysis is limited by this step (Hasselbach, 1979) it was suggested by Inesi et al. (1980), and Martonosi and Beeler (1983) that the active quaternary complex is created through 2 successive Ca^{2+} binding steps which precede and follow Mg·ATP binding.

Subsequent to the formation of the active quaternary complex, hydrolysis of ATP occurs. The terminal phosphate moeity becomes covalently bonded to the β-carboxyl group of an aspartyl residue of the enzyme (Tada and Katz, 1982). Earlier work showed that the resulting acylphosphate is an acid stable but alkaline labile complex (Makinose, 1969). During active transport the phosphoenzyme (EP) is an obligatory intermediate (Haynes, 1983) for which Ca^{2+} binding is a necessary and sufficient condition (Kanazawa et al., 1971). It is at this step that unliganded ADP dissociation from the quaternary complex occurs (Ronzani et al., 1979; Sakamoto and Tonomura, 1980; Hasselbach et al., 1981).

Translocation of Ca^{2+}

The transient state kinetics of EP formation are complex and have been studied in both the solubilised ATPase (Yamada et al., 1971; Kanazawa et al., 1971) and the fragmented SR (FSR) (Froelich and Taylor, 1975; 1976). Upon addition of ATP to both fractions, the time course of inorganic phosphate (Pi) liberation consisted of lag phase, a burst phase and a steady phase. EP formation occured without a lag phase although a prominent burst was evident. Froelich and Taylor (1975), with fSR, observed a transient decay of EP that coincides with the Pi burst. To account for this they proposed the existence of an acid-labile EP intermediate. However, when ATP hydrolysis was initiated by the addition of both Ca^{2+} and ATP (with and without Ca^{2+} preincubation) the Pi burst and EP overshoot disappeared (Takisawa and Tonomura, 1978). Since Ca^{2+} binding is not rate limiting it was suggested that the acid-labile EP is formed through, at least, two TCA-stable intermediates (Yamamoto et al., 1979). This assumption, although consistent with the proposal of sequential formation of two acid-stable EP intermediates with differing Ca^{2+} affinities(Ikemoto, 1976), is, however, based solely upon the evidence presented and the assumption that Ca^{2+} and ATP bind randomly.

EP formation in native vesicles exhibits a second power dependency upon the external concentrations of Ca^{2+} (Froehlich and Taylor, 1976). At this point the bound Ca^{2+} becomes inaccessible to external EGTA (Hasselbach and Oetliker, 1983). Fassold et al. (1981), using GTP as the reaction substrate, found that upon prior occupation of the Ca^{2+} binding sites with Ca^{2+}, Ca^{2+}/Pi ratios of up to 5 were observed. Hasselbach and Oetliker (1983) suggested that phosphorylation of protein subunits confers the property of EGTA inaccessibility without necessarily implying Ca^{2+} translocation at this stage. Such an observation may be linked to oligomeric subunit interactions (Pick and Karlish, 1980). Takisawa and Makinose (1983), however, questioned the usefulness of EGTA quench techniques, in this regard, since EGTA fails to instantaneously attenuate EP Formation and subsequent EP isomerisation and decay. The stoichiometry associated with transient Ca^{2+} and EP kinetics is, consequently, obscure (Fassold et al., 1981).

Within native vesicles, EP formation and Ca^{2+} transport were suggested to occur synchronously (Verjovski-Almeida et al., 1978) with both events apparently associated with a significant decrease in EP affinity for both Ca^{2+} and Mg^{2+} (Yamada and Tonomura, 1972). Hasselbach and Oeltiker (1983) argued, however, that this is plausible only if during translocation the affinity reduction of the enzyme for Ca^{2+}, upon initial formation of EP, occurs through 3 or 4 orders of magnitude. This implies that very high internal concentrations of Ca^{2+} would be required for EP binding during the reverse kinetic step. Ronzani et al. (1979) and The et al. (1981) indicated, though, that this is not the case since the reverse kinetic step, as monitored by ATP-ADP exchange, can occur in the absence of Ca^{2+} and Mg^{2+}. The same research group (Takisawa and Makinose, 1983) suggested that Ca^{2+} translocation does not occur upon EP formation but, rather, is coincident upon conversion of an ADP-sensitive EP to an ADP-insensitive EP. Support for this comes from comparison of energy partitioning in tight and leaky vesicles (Hasselbach et al., 1981) indicating that the energy of this isomerisation step is transfered into osmotic work and the creation of an inward Ca^{2+} gradient. Since tight vesicles can generate a Ca^{2+} gradient of 1,000 (Ca^{2+} inside: Ca^{2+} outside), the Ca^{2+} affinity of the phosphoprotein presumably decreases ($10^6 M^{-1}$ to $10^3 M^{-1}$) during translocation (Martonosi and Beeler, 1983). This suppostion is verfied through the observation that Ca^{2+} dissociation occurs prior to EP Decay (Makinose, 1973). Futhermore, as elevated luminal Ca^{2+} concentrations inhibit protein dephosphorylation (DeMeis and Vianna, 1979) then the affinity change must occur during the isomerisation between two or more EP intermediate states.

Release of Ca^{2+}

Following translocation, Ca^{2+}, at the now low affinity binding site, is released into the vesicle lumen (Chiu and Haynes, 1980). Chiu and Haynes (1980) found that the rate of release to be rapid ($t1/2 < 50ms$) although internal concentrations of K^+ ions greater than 50mM were required. The rate of Ca^{2+} dissociation is also a direct fuction of Mg^{2+} concentration and is explained by Ca^{2+}/Mg^{2+} competition for binding (Chiu and Haynes, 1980). In vitro, however, this effect is difficult to assay since Mg^{2+} effects are also associated with EP

dephosphorylation.

At this point the thermodynamically unstable high energy EP binds Mg^{2+} which is essential for EP decay (Inesi et al., 1974). Mg^{2+} binding occurs at the Ca^{2+} low affinity sites and as such the former, in this capacity, acts internally and competes with Ca^{2+} with first order kinetics (Yamada and Tonomura, 1972; Kometani and Kasai, 1980). Garrahan et al. (1976), in contrast, proposed that Ca^{2+} and Mg^{2+} binding, during EP decay, occurred at separate sites. Their assay conditions, however, included CDTA, a chelating agent, which Tada et al. (1978) suggested may have altered the kinetic properties of the EP intermediate.

More recently, the role of monovalent cations, particularly K^+ and protons, have been implicated in relation to EP decay (Ueno and Sekine, 1978; Gerdes and Moller, 1983; Haynes, 1983). Haynes (1983) proposed a model in which the high energy EP could bind Mg^{2+} or K^+ and that the respective dephosphorylations were capable of proceeding in parallel. The two possible cation-EP complexes are acid stable (Shigekawa and Dougherty, 1978) but each may be functionally different. The Mg^{2+} complex may be associated with regulating bidirectional fluxes of the enzyme since it is capable of participation in the back reaction of the pump cycle, as well as acceleration of EP decay (Hasselbach et al., 1981). The K^+ complex, on the other hand, accelerates ADP-insensitive EP formation (Shigekawa and Akowitz, 1979), the property of which is conferred by increasing luminal K^+ concentrations. The latter EP complex has been implicated in counter ion transport and the return of the translocator to the outward orientation (McKinley and Meissner, 1978). Chiu and Haynes (1980) provided strong evidence that Mg^{2+} is not counter transported with Ca^{2+} influx. Their use of 1-anilino-8-napthalensulphonate (ANS) as an index of active cation transport produced results inconsistent with 1:1 Mg^{2+}/Ca^{2+} transmembranous exchange. This finding is supported by Ueno and Sekine (1978) who failed to observe changes in intravesicular Mg^{2+} in a Ca^{2+} influx dependent mannner. KCl jump and valinomycin experments by Chiu and Haynes (1980) reinforce the tenet that K^+ is the major counter transported cation. In this regard, a role for protons was suggested by Kometani and Kasai (1980) and later included as a possibility by

Haynes (1983).

The concept of cation counter transport with active Ca^{2+} influx directly conflicts with the electrogenic transport model (Zimniak and Racker, 1978). Zimniak and Racker (1978), however, employed ANS as an index of membrane potential. This procedure was judged experimentally invalid (Haynes and Simkowitz, 1977) since ANS is more sensitive to transmembranous cation fluxes. Conversely, Kometani and Kasai (1980) argued that the vesicular swelling observed during Ca^{2+} accumulation precludes active K^+ countertransport. They maintained that passive anion transport must occur to ensure electroneutrality, although this was not assessed. Furthermore, no assessment of the water of hydration associated with each ion and it's contribution to the shrinking and swelling of vesicles was made. Different monovalent and divalent ions will cotransport different amounts of water such that Ca^{2+} accumulation may account for swelling despite a net K^+ loss.

Reverse Kinetic Cycle

Makinose and Hasselbach (1971) originally demonstrated pump reversal by the Ca^{2+} stimulated Ca^{2+}-ATPase with concomittant ATP synthesis and Ca^{2+} efflux from previously Ca^{2+} loaded native vesicles. Reconstituded vesicles have also been shown to exhibit this property (Racker, 1972; Racker et al., 1975) and the stoichiometry of the reverse process matched that of the forward reaction (Hasselbach, 1979). Since the original findings, several researchers have verified that this manner of Ca^{2+} efflux from vesicles is an ADP, Pi, and Mg^{2+} induced mechanism (Barlogie et al., 1971; Makinose, 1972; Yamada et al., 1972; Inesi et al., 1973). Furthermore, asymmetric Ca^{2+} binding to the SR membrane is an absolute requirement with a concentration gradient that is outwardly directed (Tada et al., 1978). This observation applies equally to both actively (DeMeis, 1976) and passively (Vale et al., 1976) Ca^{2+} loaded vesicles. Although Ca^{2+} efflux can occur in the absence of Mg^{2+}, ADP, and Pi (in the precence of external EGTA), the rate of efflux is considerably slower (Tada et al., 1978).

Hasselbach et al. (1981) reported that Ca^{2+} efflux via pump reversal is initiated by the incorporation of Pi which is dependent upon the concentration of free Mg^{2+}.Earlier, however,

DeMeis and Vianna (1979) suggested that trigger the for ATP synthesis and Ca^{2+} efflux is Pi transfer from EP, based on observations of the stimulatory effects of ADP (Makinose, 1972; Yamada et al., 1972). Hasselbach et al. (1981) proposed that the failure by previous attempts to observe the stimulatory effects of Mg^{2+} upon Ca^{2+} efflux may be a consequence of the low Mg^{2+} concentrations required. Despite this, the consensus view is that the acylphosphate of EP is an acid stable complex similar to that generated by the forward reaction.

Phosphorylation of the Ca^{2+}-ATPase by Pi appears capable of proceeding within a Ca^{2+} free system (Masuda and DeMeis, 1973). Futhermore, the rate of Mg^{2+} and Pi binding to the Ca^{2+} free protein is rapid (Hasselbach et al., 1981). However, asymmetric transmembranous Ca^{2+} binding considerably alters the affinity of the translocator for both Mg^{2+} and Pi (Hasselbach, 1979) with the extent of phosphorylation directly proportional to the size of the the Ca^{2+} gradient (Yamada et al., 1972).

Beil et al. (1977) reported that the subsequent step in Ca^{2+} efflux, that of Pi transfer to ADP, was not possible when vesicle membranes were leaky. Hasselbach et al. (1981) explained this on the basis that energy partitioning, which is regulated by Ca^{2+}, is altered by membrane leakiness such that EP isomerisation becomes thermodynamically unfavorable. DeMeis (1976) suggested that the Ca^{2+} potential was expended for Pi transfer from EP to ADP. This kinetic event was later suggested to be a function of Ca^{2+} occupation of the low affinity sites (DeMeis and Vianna, 1979).

Regulation of Ca^{2+}-ATPase Kinetics

Ca^{2+} Regulation

Calcium, the ion of central interest in SR Ca^{2+} transport is critically involved in the regulation of Ca^{2+}-ATPase kinetics and Ca^{2+} movements (Inesi et al., 1974; Chu et al., 1983; Meltzer and Berman, 1984). Under optimal conditions of Mg·ATP, pH and temperature, rapid Ca^{2+} influx in SR vesicles, is stimulated at Ca^{2+} concentrations above 0.01μM with maximal Ca^{2+} influx occuring in the presence of 1- 10μM Ca^{2+} (Weber et al., 1963; Hasselbach, 1979).

The maximum amount of Ca^{2+} which can be accumulated by the vesicles, in the absence of precipitating anions is approximately 100-150 nmoles.mg^{-1} of SR protein (Takenaka et al., 1982). Furthermore, the Ca^{2+}-ATPase is capable of reducing external Ca^{2+} to within 1-5 nM (Hasselbach, 1979). Since internal Ca^{2+} concentrations of between 5 and 20mM (Meltzer and Berman, 1984) can be achieved, even after accounting for Ca^{2+} binding to intraluminal sites (Ikemoto, 1975, Maclennan et al., 1972), then inward Ca^{2+} gradients can be established (Hasselbach, 1981).

During active transport, external Ca^{2+} concentrations above 29μM were found to inhibit Ca^{2+} accumulation (Chiu and Haynes, 1980). Although prior occupation of the external high affinity Ca^{2+} binding sites stabilizes the Ca^{2+}-ATPase in its influx mode (Chiu and Haynes, 1980) rising Ca^{2+} concentrations form an inhibitory Ca-ATP complex (Kanazawa et al., 1971; Hasselbach et al.,1981). Ca-ATP, under particular conditions may serve as a substrate for the Ca^{2+}-ATPase (Wakabyashi and Shigekawa, 1984; Shigekawa et al., 1983(a)) but the partial catalytic reactions were found to be considerably slower than with Mg·ATP as the substrate (Shigekawa et al., 1983(b)).

Internal Ca^{2+}, although central to the energy partitioning of the EP isomerisation, has been shown to inhibit Ca^{2+} accumulation when increased up to 20mM (Yamada et al., 1972). The mechanism by which Ca^{2+} exerts its inhibitory effect is uncertain although Ca^{2+}/Mg^{2+} competition for internal binding (and hence depressed EP decay) has been suggested (Yamamoto et al., 1979) together with the potentiation of Ca^{2+}-ATPase mediated Ca^{2+} efflux through low affinity site binding of Ca^{2+} (Hasselbach and Waas, 1982).

Mg^{2+} Regulation

Magnesuim appears to play a particularly complex role in the regulation of Ca^{2+}-ATPase functioning. The complex of Mg^{2+} and ATP is considered to be the physiological substrate for the Ca^{2+}-ATPase (Weber et al.1966; Makinose and Boll, 1979) with the cation acting, possibly, as an ionic cofactor or charge compensator for the nucleotide (Hasselbach et al., 1981). At this point Mg^{2+} may also act in accelerating an ATP dependent isomerisation of

the Ca^{2+}-ATPase upon initial binding of the Mg·ATP substrate (Champeil et al.,1983).

Mg^{2+} is thought to remain bound or in an occluded state (EDTA inaccessible) upon initial EP formation (Hasselbach and Oetliker, 1983) and has been shown to activate the reversible transphosphorylation from ATP to the enzyme (Hasselbach et al., 1981). Moreover, Mg^{2+} was shown to accelerate conversion of the ADP-sensitive to ADP-insensitive phosphoenzymes (Shigekawa and Dougherty, 1978) as well as being essential for the phosphorylation of the Ca^{2+}-ATPase by Pi (Masuda and DeMeis, 1973). Furthermore Mg^{2+} is also essential for enzyme dephosphorylation as montiored through Pi-H_2O-exchange (Kanazawa and Boyer, 1973). The range of effects described for Mg^{2+} may be effected through binding at a variety of sites or Mg^{2+} may act a single site. The research evidence in this regard is inconclusive. Makinose and Boll (1979) suggested that the activating effects of Mg^{2+} upon enzyme hydrolysis are mediated through binding at a site other than the substrate binding site. Yamada and Tonomura (1972), alternatively, suggested that Mg^{2+} may mediate rapid EP hydrolysis through binding to external Ca^{2+} sites. Shigekawa et al. (1983) concluded that the enzyme bound Mg^{2+}, which is responsible for rapid EP hydrolysis is derived from the substrate Mg·ATP. Takakuwa and Kanazawa (1982) reported the converse, however, suggesting that, during EP hydrolysis, Mg^{2+} mediates exposure of the bound Ca^{2+} to the luminal space and is derived from sources other than Mg·ATP

Millimolar concentrations of Mg^{2+} (1-5mM) are required for the stimulation of both Ca^{2+} influx and efflux, although rising concentrations of both internal and external Mg^{2+} (10mM) have been shown to inhibit influx (Froelich and Taylor, 1976). The inhibition in many cases is presumably due to Mg^{2+} competition with Ca^{2+} for binding (Yamamoto et al., 1979). Haynes (1983) suggested that rising internal Mg^{2+} may lead to the accumulation of the ADP-insensitive species of EP since Mg^{2+} is not thought to be countertransported during Ca^{2+} influx (Chiu and Haynes, 1980; Ueno and Sekine, 1978). The result of this may be inhibition of Ca^{2+} influx since the translocator could not return to the outward orientation.

ATP Regulation

The vesicular form of the Ca^{2+}-ATPase displays a non-linear dependence upon ATP for both Ca^{2+} transport and ATPase activity (Martonosi and Beeler, 1983). There is common agreement that, in this form, Mg·ATP, in addition to being the true substrate (Hasselbach et al., 1981), also exerts an activating effect upon Ca^{2+}-ATPase kinetics at higher substrate concentrations. At low Mg·ATP concentrations ATP hydrolysis increases hyperbolically with first order kinetics and reaches a plateau at 100μM. Mg·ATP concentrations beyond 0.1mM results in a secondary activating effect upon ATP hydrolysis (McIntosh and Boyer, 1983). When displayed as Lineweaver-Burke plots, a downward deviation in linearity is observed at higher substrate concentrations (Yamada and Tanomura, 1972). Consequently, two distinct Km values of 2-3μM and 500μM, respectively, are generally observed (Moller et al., 1980). Some authors (Vianna, 1975; Yates and Duance, 1976), however, reported activating effects of Mg·ATP at lower substrate concentrations (20 to 40μM). In this light Moller et al. (1980) cautioned interpretation of results derived from coupled enzyme assays in which the Ca^{2+}-ATPase reaction is not the rate limiting step particularly a low substrate concentrations.

The apparent biphasic dependence of the Ca^{2+}-ATPase kinetics upon substrate concentrations seems to indicate the presence of 2 classes of Mg·ATP binding sites (Hasselbach, 1979). Furthermore, the nature of the dependency is indicative of some negative cooperativity between the two binding sites (Moller et al., 1980). The high affinity site (Kd=2-3μM) has been identified as the catalytic site whereas the low affinity site has a regulatory function (Dupont, 1977). The precise role for the 2 sites and their interaction upon Mg·ATP binding is not fully understood. Kanazwa et al. (1971) found that the ratio of Pi formation to EP concentration was virtually independent of substrate concentrate. These authors suggested that higher substrate concentrations accelerate EP formation rather than the rate of dephosphorylation. In contrast, Froelich and Taylor (1975) suggested that high Mg·ATP concentrations accelerate dephosphorylation.

Chiu and Haynes (1980) proposed the existence of a single Mg·ATP binding site, the nucleotide affinity of which is dependent upon the cationic concentration. Pick (1982) showed that this may be the case since 2 interconvertible forms of the same Mg·ATP binding sites were suggested from their data after cationic manipulations. Earlier studies (Pick and Karlish, 1980; Pick and Bassilian, 1980), using fluorescein 5'-isothiocyanate (FITC), support this view since FITC inhibited, differentially, catalytic and regulatory site binding while indicating the presence of only one ATP binding site. Additional support for this concept was demonstrated by similar studies of ADP and Pi stimulation of Ca^{2+}-ATPase mediated Ca^{2+} efflux (Pick and Bassilian, 1980). Haynes (1983) reviewed similar observations with Na^+/K^+ ATPases (Moczdlowski and Fortes, 1981) and suggested that the ATP effect upon Ca^{2+}-ATPase functioning may be due to a specific monomeric enzyme site of variable affinity. In view of this Haynes (1983) challenged the usefulness of assumptions regarding oligomeric functional units (Hasselbach and Oetliker, 1983).

Monovalent Cation and Anion Regulation

The effects of monovalent cations and anions, specifically K^+, Cl^-, H^+ and Pi, upon SR Ca^{2+} transport are relatively obscure. The effects of K^+ are seemingly disparate since both inhibitory and facilitatory effects have been reported (Martonosi and Beeler, 1983). Both modes of action, however, appear to depend upon the concentrations of Ca^{2+}, Mg^{2+} and ATP. At suboptimal levels of divalent cation and nucleotide, K^+ renders the forward conversion of ADP-sensitive EP to ADP-insensitive EP a rate limiting step and activates the reverse reaction (DeMeis and DeMello, 1973; Chaloub and DeMeis, 1980; Ikemoto et al., 1981). This results in a loss of the secondary activating effects of ATP and an accumulation of the ADP-sensitive EP. On the otherhand, Chiu and Haynes (1980) found that, under optimal conditions of ionic and substrate concentration, K^+ (above 50mM) was required for rapid release ($t1/2 < 50ms$) of Ca^{2+} from the inwardly oriented translocator during active influx.

Associated with the disparate effects of K^+ is the divergent opinion regarding the role of membrane potentials during active Ca^{2+} transport. Zimniak and Racker (1979) found that Ca^{2+} influx, in reconstituted vesicles, generated an inside positive membrane potential which was not dissipated through valinomycin or ethanol treatment of membranes. This result partially supports the finding by Kometani and Kasai (1980) that monovalent cations (particularly K^+) are not counter transported during active Ca^{2+} influx although they suggested a possible role of Cl^- cotransport.

Chiesi and Inesi (1980) and Madeira (1980) provided evidence of proton countertransport. This is in accord with the suggestion by Haynes (1983) that increased internal proton concentrations can accelerate the return of the outwardly oriented translator. Haynes (1983) cited the work of Meissner (1973), in which external Ca^{2+} binding affinity was reduced with decreasing pH, as support for a model of monovalent cation countertransport during active Ca^{2+} influx. The Ca^{2+}/H^+ dependence of the Ca^{2+}-ATPase has been, more recently, studied and may be associated with regulation of the coupling between Ca^{2+} transport and ATP hydrolysis (Meltzer and Berman, 1984).

That discrepant opinion exists, regarding the role of monovalent ligands in Ca^{2+} transport, is likely exacerbated by the fuctional heterogenity of the SR. The need to account for this will be important, especially, since isolated vesicles have demonstrated both permeability to $K^+$and CL^- ions (McKinley and Meissner, 1978; Meissner and Young, 1980).

Ionic Permeability of SR

The membrane lipid fracton of the SR represents the primary barrier to ion fluxes (Vanderkooi and Martonosi, 1971). The significantly increased permeability to inulin subsequent to treatment of SR Vesicles with phospholipases (Duggan and Martonosi, 1970) supports this contention. The relative permeability and permeation rate constants of a variety of ionic species has been determined using isotope exchange techniques (Meissner and McKinley, 1976) and stopped flow spectrophotemetry of light scattering (Kometani and Kasai,

1978). The relative permeability of a variety of important ions is as follows; Ca^{2+}, Mn^{2+}, Mg^{2+}<gluconate$^-$, Choline$^+$, Tris$^+$ < methane sulphonate$^-$ < urea, glycerol < Li^+ < Na^+, K^+ < Cl^- < H^+. For present purposes only permeability to Ca^{2+} and monovalent cations and anions will be discussed futher.

Monovalent Cation and Anion Permeability

The significantly greater permeability of SR vesicles to monovalent cations and anions as opposed to Ca^{2+} is indicative of the presence of cation and anion channels within the SR membrane (McKinley and Meissner, 1977, 1978). Futhermore, it was suggested that the SR is heterogenous with respect to cation channels (Kometani and Kasai, 1978) and possibly anion channels (Chiu and Haynes, 1980). Two thirds of the SR vesicle population (Type I) was proposed to be monovalent cation permeable whereas the remaining one third (Type II) was suggested to be impermeable to monovalent cations (McKinley and Meissner, 1978; Chiu and Haynes, 1980). The function of these putative channels is uncertain although Martonosi (1984) suggested that they may serve to minimize osmotic and potential changes associated with Ca^{2+} accumulation and in vivo Ca^{2+} release. Kometani and Kasai (1978), Chiu and Haynes (1980), earlier, suggested that the increased permeability afforded by these channels facilitates the return of the Ca^{2+}-ATPase translocator at a cytoplasmically oriented conformation.

Ca^{2+} Permeability

With respect to membrane phospholipids, Martonosi (1984) suggested 3 mechanisms by which Ca^{2+} might traverse the membrane: passive diffusion; binding to acidic phospholipids and subsequent "flip-flop" and; Ca^{2+} penetration through lipid bilayer pores. However, the unfavorable thermodynamics of the first two mechanisms and the very slow rates of passive Ca^{2+} fluxes in liposomes as compared to SR vesicles (Vanderkooi and Martonosi, 1971) preclude a significant role for membrane phospholipids in the mechanism of Ca^{2+} flux.

The major determinant of passive transbilayer Ca^{2+} flux is likely to be the associated SR membrane proteins. This is supported by the observation that Ca^{2+}-ATPase incorporation into liposomes significantly increases Ca^{2+} permeability to a level comparable with native SR vesicles (Jilka and Martonosi, 1977). Martonosi (1984) suggested that this effect is likely due to a disordering of the immediate lipid environment around the Ca^{2+}-ATPase. A direct involvement of the translocator was dismissed on the basis that passive Ca^{2+} fluxes were found to be a direct function of the imposed Ca^{2+} gradient (Jilka and Martonosi, 1977).

Chiu and Haynes (1980), however, demonstrated biphasic responses for passive bidirectional Ca^{2+} fluxes: a fast initial phase preceded by a slow subsequent phase. The contribution of the fast phase to the total Ca^{2+} movement during influx was less than the corresponding phase during efflux. Furthermore during efflux, K^+ was found to accelerate the fast phase. These authors suggested that the Ca^{2+}-ATPase mediates passive Ca^{2+} permeability during the fast phases through a partial turnover of the pump in the steady state. The slow rate was explained by a preference of the Ca^{2+} binding site on the Ca^{2+}-ATPase for a cytoplasmic orientation.

Sorenson et al.(1980) proposed that the biphasic nature of passive Ca^{2+} fluxes may reflect two distinct populations of SR vesicle with differing Ca^{2+} permeability. That these may represent contributions from fractions derived from the cisternae and longitudinal regions of the SR is questioned by the observation (Feher and Briggs, 1982) that these two fractions possess both kinetic phases. In contrast to Chiu and Haynes (1980), Feher and Briggs (1982) found that the fast phase could be accounted for by rapid Ca^{2+} binding. When taken into account Ca^{2+} efflux was directional proportional to Ca^{2+} load. This, then, suggests simple diffusion. Gerdes et al. (1983), however, obtained a non-linear relationship between Ca^{2+} load and passive Ca^{2+} efflux. From these observations it was suggested that an oligmeric form of the Ca^{2+}-ATPase may play a role in the formation of hydrophillic negatively charged transmembrane channels through which ion exchange could occur.

Ca^{2+} Release Mechanisms

Ca^{2+} Induced - Ca^{2+} Release

Since the earlier investigations of Endo et al. (1970) the phenomenon of Ca^{2+} induced - Ca^{2+} release (CICR) has been extensively studied in cardiac (Fabiatio, 1982, 1983), skinned skeletal muscle (Endo, 1977), and in native SR vesicles (Miyamoto, 1981; Nagasaki and Kasai, 1983). CICR is characterized as requiring a particular threshold level of intraluminal Ca^{2+} (Morii and Tonomura, 1983). Below the threshold level of Ca^{2+}, increases in extraluminal Ca^{2+} serve only to increase Ca^{2+}-ATPase mediated Ca^{2+} accumulation. The threshold is not strictly defined but is considered to be a function of the relative concentrations of Mg^{2+}, ATP, and Ca^{2+} (Martonosi, 1984). Above the threshold, the rate of Ca^{2+} release is a function of the size of the transmembrane Ca^{2+} gradient. Consequently, although increasing free Mg^{2+} concentrations have been shown to inhibit CICR (Stephenson, 1982), conditions of low Mg^{2+}, high ATP and high trigger Ca^{2+} concentration are sufficient to induce Ca^{2+} release in the presence of very low intraluminal Ca^{2+} concentrations (Martonosi, 1984).

ATP has been shown to be a major modulator of the CICR mechanism (Endo, 1981). At millimolar concentrations, ATP can increase the rate of Ca^{2+} release from SR vesicles by more than 100 fold (Nagasaki and Kasai, 1983). ATP, in this capacity is thought to regulate a putative Ca^{2+} release channel that is functionally different from the Ca^{2+}-ATPase.

The activating effects of extraluminal Ca^{2+} and ATP and the inhibition by Mg^{2+} upon CICR preclude simple pump reversal as a mechanism of active Ca^{2+} release (Mitchell et al., 1984; Palade et al, 1983; Shoshan et al., 1980, 1983). Nagasaki and Kasai (1983) proposed the existence of a gated channel mechanism in which the binding of ATP converts the channel from an inactive to an active form. Without affecting the gating mechanism, then, ATP appears to increase the channel conductance. According to the proposed model, modulators such as Mg^{2+} and caffeine seem to affect only the gating mechanism of the channel (Nagasaki and Kasai, 1983). The inhibitition created by Mg^{2+} is thought to be mediated through Mg^{2+} competition for Ca^{2+} binding sites. Caffeine, on the other hand, is thought to bind at a separate site and

appears to increase the affinity for Ca^{2+} at the Ca^{2+} binding sites (Kirino et al., 1983).

Whether CICR represents the physiological mechanism mediating excitation-contraction coupling is a matter of considerable debate (Endo, 1977; Fabiato, 1982, 1983; Frank, 1982; Stephenson, 1982; Winegrad, 1982; Martonosi, 1984). For both skeletal and cardiac muscle arguments exist for and against such a mechanism although, in the case of the latter, experimental findings provide strong indication that this process may have great physiological import (Fabiato and Fabiato, 1979; Fabiato, 1983; Kirino et al, 1983). There is no doubt that CICR can be demonstrated using isolated muscle preparations (Ford and Podolsky, 1970; Endo, 1977; Fabiato, 1981) and native SR vesicles (Ohnishi, 1979; Kirino and Shimizu, 1982; Kirino et al, 1983; Morii and Tonomura, 1983; Nagasaki and Kasai, 1983). However, the major arguments directed against the plausibility of this mechanism focus upon the fact that (for skeletal muscle at least) a) CICR has only been induced under unphysiologically exaggerated conditons (Endo, 1977) b) CICR does not produce maximal contractions in spite of the potential for positive feedback (Winegrad, 1982) and c) the rate of trans-sarcolemmal Ca^{2+} flux is too slow too account for the rate of E-C coupling (Hill, 1949; Ashcroft and Stanfield, 1981; Stanfield and Ashcroft, 1982). Fabiato (1983) argued against the likelihood that a process present in skeletal muscle would be in cardiac muscle in view of the similarity of the two stimulated muscle types.

With respect to skeletal muscle, Endo (1977) argued against a CICR since at physiological Mg^{2+} concentrations (5mM) the level of free Ca^{2+} required to induce Ca^{2+} release is too high to occur during physiological stimulation. Furthermore, Endo (1977) demostrated that CICR could only be observed under conditions of high intraluminal SR Ca^{2+} load. Stephenson (1982),however, re-evaluated the methodologies employed in earlier studies. Stephenson (1982) argued that assumptions regarding myofilament space buffering of Ca^{2+} with EGTA were inadequate. Consequently, high isolation bath Ca^{2+} concentrations cannot be equated with myofilament space Ca^{2+} concentrations. Therefore, Endo's (1977) foregoing argument may not be strictly true. Stephenson's (1982) argument is supported by the

observation of caffeine contractures (which are Ca^{2+} dependent) occuring even in the presence of high bath EGTA.

With respect to cardiac muscle, the foregoing limitations do not seem to apply. Fabiato and Fabiato (1975) showed that neither heavy preloading of the SR nor a free Ca^{2+} concentration higher than the threshold for contraction of the myofilaments is required to induce a Ca^{2+} release from the SR of skinned cardiac cells. Furthermore, it was shown that an increase in free Mg^{2+} concentration up to a presumed physiological level (3.16mM) actually potentiated the amplitude of the Ca^{2+} release (Fabiato, 1981). Fabiato (1983) suggested that the gating stimulus is more a function of the rate of change of the free Ca^{2+} rather than the Ca^{2+} concentration per se. It was suggested that failure to account for this possiblity in previous studies with skeletal muscle preparations explain why CICR has been poorly demonstrated in this model. Fabiato (1982) demonstrated this in cat caudofemoralis muscle under conditions of low Ca^{2+} pre-loading, physiological Mg^{2+} (3.16mM) and a low concentration of trigger Ca^{2+} (1μM Ca^{2+} delivered in 200 milliseconds).

Ion Substitution Induced - Ca^{2+} Release

The concept of release mediated directly through depolarization of the SR membrane was initially investigated by Constantin and Podlosky (1967). The application of KCl(Cl^- addition) and Tris Proprionate (K^+ withdrawal) to Natori (1954) fibres (oil skinned) bathed in low Cl^- was shown to initiate contraction. Similar studies were conducted by Endo et al. (1970) and, since the mechanical response was similar for Cl^- addition as it was for K^+ withdrawl, it was suggested that a potential change across the SR membrane initiated the observed Ca^{2+} release. However, the principle of this procedure has been challenged on the basis that Cl^- entry into the SR lumen may be associated with cotransport of H_2O and concomittant osmotic swelling of the reticular network (Miyamoto and Kasai, 1979; Meissner and McKinley, 1976). Although the contractile response was shown to be mediated via increases in myoplasmic Ca^{2+} (Endo and Blinks, 1973) the release of Ca^{2+} was considered a consequence of either increased permeability to Ca^{2+} or osmotic lysis. In order to circumvent the problem of H_2O shifts,

Mobley (1979) performed ion substitution experiments with skinned fibers while maintaining a constant [K][Cl] product. This, supposedly, would have minimised H_2O movements. Cl^- addition produced contractures under these conditions although the magnitude of the response was diminished.

Although Ca^{2+} release induced by ion substitution is generally accepted as an observation (Martonosi, 1984), the theoretical basis of the mechanism is complicated by the potential interaction of several effects. Endo (1977) proposed that Cl^- substitution may initiate a transient local rise in Ca^{2+} concentration which itself may result in a CICR. This model, however, is incomplete since it does not account for the graded nature of Cl^- induced contractures (Winegrad, 1982). Futhermore, if Cl^- entry is associated with similar H_2O shifts then presumably this will cause an effective decrease in the internal and an increase in the external Ca^{2+} concentration. Such conditions may create an unfavorable enviroment for CICR since the absolute change will be greater intraluminally than extraluminally.

Central to this issue of ion substitution induced - Ca^{2+} release is whether or not membrane polarity is associated with Ca^{2+} release and whether or not Cl^- induces polarity or depolarises a pre-existing membrane potential. With respect to active Ca^{2+}-uptake, Zimniak and Racker(1978) have shown, using reconstituted vesicles that the Ca^{2+}-ATPase is inhibited by an inside positive potential. Kometani and Kasai (1978) and Haynes (1982), however, demonstrated that, physiologically, membrane potentials were dissipated rapidly by the counter and cotransport of monovalent cations and anions, respectively. Alternatively, with respect to Ca^{2+} release, Oetliker (1982) has shown that membrane potentials are generated in response to Ca^{2+} release in living muscle. Beeler et al. (1979, 1981) argued that the possibility of pre-existing SR membrane potentials is incompatible with the rapid permeability of the membrane to Na^+, K^+ and Cl^-. This, however, may not be entirely valid since one third of the isolated SR vesicle fraction was shown to be impermeable to these ions (McKinley and Meissener, 1977, 1978). Futher evidence against the existence of resting membrane potentials is provided by Somlyo et al. (1981) who found little evidence of asymmetric bilayer distribution

of Na^+, K^+ and Cl^-. Their electron probe microanalysis technique only measures elemental species and not the concentrations of their respective ionic forms. Additionally, the critics of Cl^- induced Ca^{2+} release do not consider the potential contribution of a Donnan pressure model which proposes the existence of fixed intraluminal charges (Kasai, 1980; Yamamoto and Kasai, 1980) possibly established by phospholipid bilayer asymmetry and/or extrinsic proteins.

Recent studies (Campbell and Shamoo, 1980; Kasai and Miyamoto, 1978; Caswell and Brandt, 1981) demostrated that Cl^- induced Ca^{2+} release may not be entirely osmotic in nature. Native vesicle were fractionated into "light" and "heavy" populations which were derived, respectively, from the longitudinal and cisternal regions of the SR. Incubation of vesicles in sucrose (an osmotic buffer) and transfered from an osmotically inactive solution (Potassium Methanesulphate or Potassium Gluconate) to an osmotically active solution (KCl) prevented release of Ca^{2+} from the light fraction but cisternal release of Ca^{2+} was unaffected in relation to the results obtained in the absence of sucrose. These results supported the contention that the cisternal fraction is primarily responsible for release and that ion-induced Ca^{2+} release from the heavy fraction may only have a small osmotic basis (Campbell and Shamoo, 1980). Koshita et al. (1982), on the other hand, did not demonstrate a differential response by the different SR fractions with and without sucrose pre-incubation. They supported the concept of osmotically induced Ca^{2+} release. However, closer inspection of their isolation procedure and the data for Ca^{2+}-dependent and Mg^{2+}-dependent ATPase strongly suggests a very high (50%) mitochondrial contamination

It is interesting to note that the proponents of an osmotic basis for ion-induced Ca^{2+} release only report partially on the data obtained by Kometani and Kasai (1978). These authors, indeed, observed osmotic swelling by vesicles upon addition of KCl. However, a consistently neglected observation is the rapid shrinking of vesicles due to extrusion of H_2O caused by the instantaneous rise in osmotic pressure upon KCl addition. These events occurred prior to the transmembrane movement of solute accompanied by the inflow of H_2O. The concomittant swelling, therefore, represented a return to normal vesicle size which was not

complete even within 20 seconds after KCl addition.

Excitation-Contraction Coupling and Fatigue

The role of the sacroplasmic reticulum (SR) in muscle fatigue has been assessed from cryo-ultramicrotomy studies of ionic composition within subcellular compartments (Somlyo et al., 1978; Gonzales-Serratos et al., 1978; Sembrowich et al., 1980, 1983). Subsequent electron probe analysis has commonly revealed extensive ionic imbalance and pronounced vacuolation of the T-tubules (Gonzales-Serratos et al., 1978). In particular, elevated Ca^{2+} contents of the SR lumen have been observed in both fast and slow twitch muscles (Sembrowich et al., 1980) and in frog semitendonosus terminal cisternae (Somlyo et al., 1978). The elevated Ca^{2+} has been suggested to disrupt Ca^{2+} transport (Gonzales-Serratos et al., 1978) and possibly, to uncouple the action potential from muscle contraction (Scales and Sabadini, 1979; Bianchi and Narayan, 1982).

That the Ca^{2+}-ATPase may be involved in the development of muscle fatigue is indicated from in vitro studies of Ca^{2+} preciptating anion (oxalate or Pi) supported accumulation of Ca^{2+} (Ca^{2+}-loading). Sembrowich and Gollnick (1977), Hashimoto et al. (1978) and Fitts et al. (1979, 1982) reported depresed Ca^{2+}loading in native SR vesicles from rats chronically exercised to exhaustion. The approximate 20% reductions in Ca^{2+} loading were paralleled by similar depressions in the activities of the Ca^{2+}-ATPase (Sembrowich and Gollnick, 1977; Belcastro et al., 1981). These findings would appear to corroborate the proportional relationship between the rate constant of muscle relaxation and the rate of Ca^{2+}-influx (Blinks et al., 1978). Together these observations provide tentative explanation for the depressed rate of relaxation at exhaustion (Dawson et al., 1980).

Belcastro et al. (1981) also reported depression of Ca^{2+}-ATPase activities in fast twitch skeletal muscle from rats chronically run to exhaustion. They suggested that the generalized depression of both Ca^{2+}-loading and ATP hydrolysis may be related to depressed Ca^{2+} binding to external high affinity sites. Chiu and Haynes (1980) and Pick and Karlish (1980, 1982)

showed that prior occupation of the high affinity Ca^{2+} binding sites stabilizes the translocator in its influx mode. Conversely, although Ca^{2+} participates in regulating energy partitioning of the Ca^{2+}-ATPase kinetics (DeMeis and Carvalho, 1974; Hasselbach et al., 1981), rising internal Ca^{2+} concentrations (2-20mM) have been shown to inhibit Ca^{2+}-loading (Yamada et al., 1972) through inhibition of translocator enzyme dephosphorylation (Haynes, 1983). Conceivably, in vivo intraluminal SR Ca^{2+} accumulation at the onset of fatigue, as indicated by Sembrowich et al. (1980, 1983), may disrupt Ca^{2+} transport via this mechanism. These observations, though, are incompatible with the in vitro data (Fitts et al., 1982) which indicate depressed initial rates of and potential for Ca^{2+}-loading by the SR at exhaustion. Establishing a cause-effect relationship on the basis of these observations is difficult, moreover, as the relative contributions of the various intraluminal Ca^{2+} compartments to the regulation of Ca^{2+}-influx kinetics remains uncertain (Chu et al., 1983; Feher and Briggs, 1983). Furthermore, since electron probe analysis fails to 1) distinguish amongst bound, free and precipitated Ca^{2+} and 2) equate increases in elemental contents with changes in ionic concentration, the interpretation of these data is limited. The latter concern, often neglected, is valid since fatigue via tetany resulted in a significant increase in total fibre volume (Gonzales-Serratos et al., 1978). Elevated intraluminal Ca^{2+} contents, therefore, need not, necessarily, reflect elevated Ca^{2+} concentrations.

Progress towards the elucidation of SR dysfunction at exhaustion may be hindered by the persistent use of oxalate or phosphate supported influx systems. Briggs et al. (1983) indicated that efflux kinetics, which appear to be mediated more by pathways other than the Ca^{2+}-ATPase (Mitchell et al., 1984; Palade et al., 1983; Shoshan et al., 1980, 1983), may in large part, determine steady state loading in oxalate supported systems. Feher and Briggs (1980) suggested that assumptions of rapid calcium oxalate crystallisation kinetics (Makinose and Hasselbach, 1965) may be tenuous since incomplete clamping of intraluminal Ca^{2+}, with variations in membrane permeability to Ca^{2+} in skeletal muscle SR, has been demonstrated (Katz et al., 1977; Feher and Briggs, 1980). Current methodologies, therefore may mask

possible aberrant Ca^{2+} flux kinetics in the SR of fatigued muscle.

The decline in intramuscular pH associated with fatigue (Gonzales-Serratos et al., 1978; Dawson, 1978, 1980) has also been suggested to affect optimal rates of Ca^{2+} transport (Edman and Maltiazzi, 1981; Nassar-Gentina et al., 1978). This is supported by, a) the proportional relationship between the rate constant of muscle relxation and the rate of Ca^{2+}-uptake (Blinks et al., 1978) together with, b) the prolonged relaxation rates observed with a decline in intramuscular pH (Dawson, 1980). Fabiato and Fabiato (1978), using frog semitendonosus muscle, suggested, however, that mild acidosis may increase Ca^{2+}-accumulation although a large acidosis (pH = 6.2) may have a depressant effect. Depressed SR function may, as consequence, be mediated through altered Ca^{2+} affinity for cytoplasmic binding. Although the work of Noble et al. (1980) does not support this, it is well established that decreasing pH decreases external Ca^{2+} binding affinity (Meissener, 1973; Verjovksi-Almeida and DeMeis, 1977; Haynes, 1983). Moreover, the potential for this is hard to ignore since a fall in pH from 7 to 6 induces a 10-fold reduction in Ca^{2+} binding affinity (Pick and Karlish, 1982). Thus the reduced binding affinity may be associated with the intracellular redistribution and imbalance of Ca^{2+} (Somlyo et al., 1978).

Appendix B - Raw Data

Table A: Raw Data for body Weight and Running times

Group Control	Weight(g)	Group Exhausted	Weight(g)	Time to Exhaustion(min)
	215		214	125
	223		246	130
	230		226	129
	265		210	134
	250		248	115

Table B: Raw Data for Microsomal Protein Yield ($mg.g^{-1}$).

Control	Exhausted
1.61	0.94
1.57	1.42
1.27	1.31
1.10	1.64
1.84	1.77

Table C: Raw Data for SR Ca^{2+}-uptake (nmol Ca^{2+}.mg SR $prot^{-1}$) at 1, 2, and 3 Minutes Incubation.

Group	1min	2min	3min
Control	28.3	28.0	37.5
	27.4	28.3	30.5
	32.1	30.4	38.8
	27.4	39.5	40.5
	39.9	38.7	37.7
	32.3	28.1	38.4
	37.9	59.9	39.7
	50.8	44.8	35.9
	40.9	52.4	43.5
	44.8	33.3	43.1
Exhausted	41.0	42.1	41.7
	37.8	35.5	32.9
	30.8	42.6	52.6
	32.6	41.9	36.5
	49.7	50.8	51.2
	44.2	48.5	57.1
	50.5	48.5	30.1
	42.9	41.2	37.2
	106.7	85.5	71.8
	75.3	86.6	79.8

Table D: Raw Data for Ca^{2+}-loading (nmol Ca^{2+}.mg SR $prot^{-1}$) at 1, 2, and 3 Minutes Incubation.

Group			
Control	1min	2min	3min
	733.9	1102.4	1300.7
	1099.0	1518.0	1684.0
	866.2	1378.0	1526.0
	1056.6	1435.6	1585.2
	721.6	1218.5	1433.0
Exhausted	861.0	1205.7	1307.8
	953.4	1376.3	1406.0
	1163.0	1638.3	1704.0
	991.9	1426.9	1589.8
	837.1	1493.1	1779.9

Table E: Raw Data for Ca^{2+}-release (nmol Ca^{2+}.mg SR $prot^{-1}$).

Amount of Ca^{2+} released expressed as a difference between Ca^{2+} remaining on filters after KGluconate and KCl washes.

Group	Post-KGluconate	Post KCl	Ca^{2+} released
Control	22.7	0.0	22.7
	12.5	0.0	12.5
	43.4	2.7	40.7
	35.5	3.3	32.2
	58.6	18.1	40.5
	56.5	15.0	41.5
	73.8	28.2	45.6
	65.5	24.6	40.6
	76.9	62.6	14.3
	87.7	65.6	22.1
Exhausted	13.6	1.0	12.6
	31.4	0.0	31.4
	40.8	0.0	40.8
	50.7	0.0	50.7
	85.8	28.4	57.4
	79.4	29.6	49.8
	80.7	31.1	49.6
	83.7	28.8	54.9
	66.7	12.3	54.4
	73.2	11.4	61.8

Table F: Raw Data for Ca^{2+}-release (nmol Ca^{2+}.mg SR $prot^{-1}$)

Amount of Ca^{2+} released expressed as a difference between Ca^{2+} remaining on filters after KGluconate and KCl washes containing sucrose.

Group	Post-KGluconate	Post KCl	Ca^{2+} released
Control	112.7	67.1	45.6
	117.8	65.5	52.3
	150.0	104.5	45.5
	145.5	129.2	16.3
	142.4	118.7	23.7
	148.0	125.6	22.4
	191.4	163.4	28.0
	202.4	165.2	37.2
Exhausted	141.5	80.9	60.6
	125.6	82.6	43.0
	186.9	129.2	57.7
	175.8	129.0	46.8
	146.1	117.7	28.4
	138.7	123.4	15.3
	205.9	106.6	99.3
	201.9	106.1	95.8

Table G: Raw Data for ATP hydrolysis within a range of ATP concentrations.

Group	[ATP] (mM)	Raw Data				
Control	**0.075**	0.082	0.087	0.086	0.084	0.087
	0.10	0.108	0.102	0.086	0.106	0.100
	0.25	0.236	0.234	0.235	0.243	0.219
	0.50	0.437	0.430	0.457	0.437	0.403
	1.00	0.796	0.803	0.776	0.795	0.758
	2.50	1.226	1.290	1.267	1.147	1.301
	5.00	1.380	1.550	1.478	1.323	1.557
Exhaust	**0.075**	0.087	0.079	0.082	0.084	0.083
	0.10	0.112	0.102	0.080	0.106	0.098
	0.25	0.227	0.226	0.220	0.236	0.215
	0.50	0.437	0.411	0.427	0.442	0.388
	1.00	0.761	0.743	0.716	0.789	0.726
	2.50	1.075	1.127	1.024	1.120	1.105
	5.00	1.180	1.308	1.288	1.286	1.316

Table H: Summary of Ca^{2+}-uptake values (nmol Ca^{2+}.mg $prot^{-1}$) for C and E at each incubation time point (mean±sem).

Incubation time	Control	Exhaust
1 minute	36.18±2.5	51.15±7.3
2 minutes	38.33±3.5	52.32±5.8
3 minutes	38.56±1.2	49.07±5.3

Table I: Summary of Ca^{2+}-loading values (nmol Ca^{2+}.mg $prot^{-1}$) for C and E at each incubation time point (mean±sem).

Incubation time	Control	Exhaust
1 minute	895.5±78	961.3±58
2 minutes	1330.5±75	1428.6±71.
3 minutes	1505.8±66	1557.5±87

Appendix C - Statistical Procedures

Table A: Two-way ANOVA summary of Ca^{2+}-accumulation by C and E at all incubation times

Source of Variation	Sum of Squares	D.F.	Mean Squares	F Ratio	Prob
A	0.3381E+02	2	16.91	0.075	0.928
B	0.2596E+04	1	2596.50	11.524	0.001
AB	0.5506E+02	2	27.53	0.122	0.885
ERROR	0.1217E+05	54	225.31		
TOTAL	0.1485E+05	59	251.73		

Table B: Two-way ANOVA summary of Ca^{2+}-uptake by C and E at all incubation times.

Source of Variation	Sum of Squares	D.F.	Mean Squares	F Ratio	Prob
A	0.1969E+07	2	984500.00	36.45	0.000
B	0.3872E+05	1	38720.00	1.43	0.243
AB	0.2816E+04	2	1408.00	0.052	0.949
ERROR	0.6481E+06	24	27002.66		
TOTAL	0.2658E+07	29	91664.50		

A=Incubation time(1, 2, or 3 mins); B=Groups(C or E)

Table C: Two-way ANOVA summary of

Ca^{2+}-release by C and E.

Source of Variation	Sum of Squares	D.F.	Mean Squares	F Ratio	Prob
A	0.1503E+05	1	1503.10	29.91	0.000
B	0.5078E+00	1	0.51	0.001	0.975
AB	0.5684E+03	1	568.38	1.31	0.295
ERROR	0.1809E+05	36	502.57		
TOTAL	0.3369E+05	39	863.91		

Table D: Two-way ANOVA summary of

Ca^{2+}-release by C and E (sucrose).

Source of Variation	Sum of Squares	D.F.	Mean Squares	F Ratio	Prob
A	0.1611E+05	1	16101.31	17.13	0.000
B	0.7143E+02	1	74.13	0.08	0.781
AB	0.9668E+03	1	966.75	1.03	0.319
ERROR	0.2633E+05	28	940.36		
TOTAL	0.4348E+05	31	1402.49		

A=Wash Media (KCl or KGluconate); B=Groups (C or E)

Table E: T-Test Summary of Vmax and Km values obtained for C and E after ATP hydrolysis.

Vmax t-statistic = 3.3018

Deg of Freedom = 8

Km statistic = 2.6860

Deg of Freedom = 8

Appendix D - Biochemical procedures and chemicals

SR Isolation Technique.

Reagents and Chemicals

1. Homogenizing Medium A (pH 6.8 at 4 °C) in mM:
   a. 100 KCl
   b. 20 Tris-Maleate
   c. 1 PMSF
2. Wash Buffer B (pH 6.8 at 4 °C) in mM:
   a. 600 KCl
   b. 20 Tris-Maleate
   c. 1 PMSF
   d. 10 NaN_3
3. Suspension Buffer C (pH 7.0 at 30 °C) in mM:
   a. 100 KCl
   b. 20 Tris-Maleate

Procedure

1. The tissue was homogenized for 2 x 10 second intervals at a setting of 8 on a Polytron Tissue Homogenizer (PT-10) in 10 volumes of cold Buffer A.
2. The homogenate was centrifuged at 1,000 x g for 20 minutes at 4°C (IEC B-20A Refrigerated Centrifuge).
3. The resultant supernatant was aspirated and centrifuged at 9,000 x g for 15 minutes at 1°C.
4. The resultant supernatant was aspirated and centrifuged at 15,000 x g for 20 minutes..
5. The resultant supernatant was carefully aspirated and centrifuged at 37,000 x g for 30 minutes.
6. The resultant supernatant was discarded and the pellet resuspended in 3 volumes of cold

Buffer B. This and all subsequent resuspensions were performed with 4-5 slow strokes of a teflon pestle in a glass homogenizer to insure the functional integrity of the fragmented vesicles. The resuspension was centrifuged at 37,000 x g for 20 minutes.

7. The resultant pellet was resuspended in 3 volumes of cold Buffer B and centrifuged at 37,000 x g for 20 minutes.
8. The resultant pellet was resuspended in 3 volumes of cold Buffer B and centrifuged at 37,000 x g for 20 minutes.
9. The resultant pellet was resuspended in 1.5 volumes of cold Buffer C. The suspension was used immediately after protein determination (1.5 hrs).

Protein Assay.

Reagents and Chemicals

1. Stock Solution A1:
   a. 2% Na_2CO_3
   b. 0.1N NaOH
2. Stock Solution A2:
   a. 10% SDS (fresh daily)
3. Stock Solution B1:
   a. 1% $CuSO_4 \cdot 5H_20$
4. Stock Solution B2:
   a. 2% $NaKC_4H_4O_6$
5. Solution C:
   a. 50.0 ml A1
   b. 5.0 ml A2
   c. 0.5 ml B1
   d. 0.5 ml B2
6. Folin Reagent (1N): 1 to 1 (v/v) distilled H_2O

Standard Curve

BSA (ml)	H_2O (ml)	Conc A ($mg.ml^{-}$)	Conc B ($mg.ml^{-}$)
0	0.500	0	0
0.05	0.45	0.10	0.02
0.10	0.40	0.20	0.04
0.15	0.35	0.30	0.06
0.20	0.30	0.40	0.08
0.25	0.25	0.50	0.10

Procedure

1. 2 x 100 mml aliquots of each concentration A were transfered to duplicate tubes containing 0.4ml H_2O to produce a protein suspension of concentration B
2. 3 ml of solution C was added to each and allowed to sit for atleast 10 mins.
3. 0.3 ml of Folin's reagent was then added while vortexing.The solution was allowed to stand for atleast 30 mins.
4. Spectrophotometric measurements were taken at 750 nm.

Ca^{2+}-uptake / Ca^{2+}-accumulation.

Reagents and Chemicals

1. Transport Medium (pH7.0 at 30°C) in mM:
   a. 100 KCL
   b. 40 Tris-Maleate
   c. 5 $MgCl_2$
   d. 0.050 $^{45}CaCl_2$ (specific activity= 10,000 $dpm.nmol^{-1}$)
   e. 5 Na Oxalate (for Ca^{2+}-Uptake)
2. $Mg{\cdot}ATP^{2-}$ (200mM; pH 7.0 at 30°C)
   a. A 50μl aliquot was added to 1.9 mls of transport medium. Therefore, final concentration of $Mg{\cdot}ATP^{2-}$ was 5mM.

Procedure

1. A 50μl of SR protein (1 $mg.ml^{-1}$) was pre-incubated in 1.9 ml of Transport medium for 3 mins. This gave a final protein concentration of 25$\mu g.ml^{-1}$. Duplicate tubes were prepared.
2. A 50μl aliquot of $Mg{\cdot}ATP^{2-}$ was added after the pre-incubation period in order to initiate Ca^{2+} transport. Control tubes without $Mg{\cdot}ATP^{2-}$ were run with each sample to assess ATP independent Ca^{2+} binding.
3. At 1,2, and 3 minute intervals 500μl aliquots were drawn and vacuum filtered across pre-moistened millipore (HAWP 0.45μ) filters.
4. Immediately following filtration the filters were washed with 2 volumes of ice-cold distilled-deionized H_2O.
5. Filters were then dried and dissolved in 10 mls Bray's scintillation cocktail.
6. Samples were counted for radioactivity upon a Beckman LS 7800.
7. Ca^{2+} loading of vesicles was calculated in relation to the dpm counted on the filter, the known specific activity of the Ca^{2+}, and the amount of protein in a 500μl aliquot (12.5μg).

Ca^{2+} Release

Reagents and Chemicals

1. Transport Media (pH 6.8 at 30°C) in mM:
   a. 200 K Gluconate or KCl
   b. 10 HEPES buffer
   c. 1 $MgCl_2$
   d. 0.05 $^{45}CaCl_2$ (specific activity = 10,000 dpm.mole^{-1})
   e. 200 Sucrose (included for specific conditions)

   Therefore 4 variations of transport media were prepared: K Gluconate with sucrose; K Gluconate no sucrose; KCl with sucrose; and KCl no sucrose.
2. Mg·ATP^{2-} (200mM: pH 6.8 at 30°C)
3. Wash Media (pH 6.8 at 30°C) in mM:
   a. 200 K Gluconate or KCl
   b. 10 HEPES buffer
   c. 1 $MgCl_2$
   d. 200 sucrose (included for specific conditions)

Procedure

1. A 50μl aliquot of SR protein (1mg.ml^{-1}) was pre-incubated for 45 mins at room temperature to allow permeation of sucrose. This was done in duplicate for each of the 4 medium variations (1.9 mls).
2. The media were then pre-incubated at 30°C for 3 mins.
3. A 50μl aliquot of 5mM Mg·ATP^{2-} was added after the second pre-incubation in order to initiate Ca^{2+} transport.
4. After 3 mins a 500μl aliquot was drawn and vacuum filtered across pre-moistened millipore (HAWP 0·45μ) filters.
5. Immediately following filtration the filters were washed with 1 volume of wash buffer. One of the filtered duplicates was washed with K Gluconate containing wash buffer, the other

with KCl containing wash buffer. Sucrose containing wash buffers were used for sucrose containing reaction buffers.

6. Filters were then washed with 2 volumes of ice-cold distilled-deionized H_2O.
7. Filters were then prepared and counted as for Ca^{2+}-uptake / Ca^{2+}-accumulation.

SR ATPase Assay.

Reagents and Chemicals

1. Reaction medium (pH 7.0 at 30 °C)
   a. 100 KCl
   b. 40 Tris-maleate
   c. 5 $MgCl_2$
   d. 0.05 $CaCl_2$
2. 200mM $Mg{\cdot}ATP^{2-}$ (pH 7.0 at 30°C)
3. 12% TCA

Procedure

1. The preparation of SR was diluted to 1.00 $mg.ml^{-1}$.
2. Duplicate tubes, each containing 0.925 mls of reaction medium were prepared. One set of duplicate tubes (X tube) was initialy quenched with 1 ml cold TCA.
3. $Mg{\cdot}ATP^{2-}$ was prediluted to give a range of final concentrations in reaction tube. These were as follows: 75μM; 100μM; 250μM; 500μM; 1mM; 2.5mM; and 5.0mM.
4. A 50μl aliquot of SR protein was added to the reaction medium and pre-incubated for 5 mins in a water bath set at 30°C.
5. A 25μl aliquot of $Mg{\cdot}ATP^{2-}$ was then added to initiate the reaction which was allowed to run for 10 mins.
6. The reaction in each tube was quenched with 1ml ice cold 12% TCA :each tube was immediately placed on ice for 10 mins.
7. Tubes were then centrifuged at 1,000 x g at 4°C for 10 mins to sediment the precipitated protein.
8. Aliquots of the supernatant were then drawn and used in the phosphate determination assay.

Phosphate Assay

Reagents and Chemicals

1. 10 N Sulphuric acid
2. Ammonium Molybdate-Ferrous Sulphate solution:
   a. 0.5g ammonium molybdate was dissolved in 5 mls 10N H_2SO_4.
   b. After complete dissolution volume was raised to 30 mls with distilled-deionized H_2O.
   c. 2.5g Ferrous Sulphate was then added; volume then made up to 50 mls.

Procedure

1. Phosphate standard and blank were prepared
   a. Phosphate standard: 1.45ml TCA + 50μl phosphate stock (1mM)
   b. Phosphate blank: 1.5ml TCA
2. 2 x 0.5ml of supernatant from ATPase assay were drawn from the X and E (experimental) tubes and placed in tubes containing 1ml 12% TCA (ice-cold).
3. 1ml of ammonium molybdate-ferrous sulphate solution was added to all tubes. This solution was allowed to sit at room temperature for 10 mins.
4. Samples were read at 700nm on a Pye-Unicam P8800 spectrophotometer.
5. ATPase activity (mM $Pi.mg^{-1}.min^{-1}$.) was calculated using the following formula; activity = 4×0.5 ((E OD - X OD).STD $OD^{-1}.0.05^{-1}.10^{-1}$)) where;
   a. 4 = Protein dilution factor
   b. 0.5 = Pi concentration in standard
   c. 0.05 = Protein concentration in reaction tube
   d. 10 = Time of reaction (mins)

List and Source of Chemicals

Adenosine 5'Triphosphate (disodium salt)	Sigma
Ammonium molybdate	BDH
Bovine Serum Albumin	Sigma
Bray's Scintillation Cocktail	Isolab
Calcium Chloride	Fisher
^{45}Calcium Chloride	NEM
Cupric Sulfate	Fisher
Ferrous Sulphate	BDH
Folin & Ciocalteu's Phenol Reagent	Fisher
HEPES	Sigma
Magnesium Chloride	Sigma
PMSF	Sigma
Potassium Chloride	Sigma
Potassium Gluconate	BDH
Sodium Azide	BDH
Sodium Carbonate Anhydrate	BDH
Sodium Dodecal Sulphate	BDH
Sodium Hydroxide	Fisher
Sodium Oxalate	BDH
Sodium Phosphate Dibasic	Fisher
Sodium Tartrate	Fisher
Sodium Sulphate	Fisher
Sucrose	Fisher
Sulfuric Acid	Baker
TCA	Fisher
Tris Maleate	Sigma